Master of Sciences in Bioengineering

Immobilization of Laccase over carbon nanotubes for biocatalysis applications

Master's Thesis

by

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performed at

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DECLARATION

It is declared on path that this work is original and that all non-original contributions have been properly referenced with the identification of the source.

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It is with great pride and pleasure that I present the following work. However, in spite of assuming full responsibility, none of this would be achieved without the unconditional support of certain people.

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ABSTRACT

The use of oxidative enzymes has received great attention due to their efficiency and selectivity. Laccase is an oxidative enzyme that is used to catalyze a wide range of phenolic compounds. Laccase is increasingly used in oxidative processes, such as: oxygen delignification, color removal, bioremediation, degradation of polycyclic aromatic hydrocarbons (PAH), biosensors, biofuel cells, among others. However, the low stability in the free form and the impossibility of reuse has limited its application at industrial level. Thus, the immobilization of laccase is an effective method of continuously applying oxidation reactions.

In this work, laccase was immobilized on multi-walled carbon nanotubes (MWCNTs) modified using different functionalization approaches: with i) carboxylic acid; ii) 3-aminopropyl)triethoxysilane (APTS); iii) glutaraldehyde; iv) both APTS and glutaraldehyde; and v) N-ethyl-N-(3-(dimethylamino)propyl) carbodiimide hydrochloride and N-hydroxysuccinimide (EDC/NHS). The main objective was to understand the behavior of the enzyme when immobilized on functionalized MWCNTs and find out the more efficient functionalization method. The enzyme-MWCNT conjugate with enhanced immobilization efficiency, catalytic activity, thermal stability and reutilization performance towards ABTS (2.2'-azino-bis(3-ethylbenzathiazoline-6-sulfonic) acid) oxidation was selected for being used for the biocatalytic oxidation of phenol.

The best performance dealing with laccase immobilization was obtained for MWCNTs treated with EDC/NHS (N-ethyl-N-(3-(dimethylamino)propyl) carbodiimide hydrochloride/N-hydroxysuccinimide). The obtained values of immobilization yield and recovered activity were 99.8% and 20.4% respectively. Regarding reutilization tests, the best performing materials were also those treated with EDC/NHS, with an immobilized laccase activity of 65% after 5 cycles of reutilization. It was also noticed that the immobilization process using this treatment promoted an increase in laccase stability, protecting it from thermal denaturation comparing with free laccase.

The characterization of MWCNTs using FTIR-ATR demonstrated that functionalization with EDC/NHS generated the introduction of functional groups, which promote the covalent immobilization of laccase on MWCNTs surface.

The biocatalytic performance of the immobilized laccase was evaluated using phenol as model compound. Free laccase and immobilized laccase were able to degrade phenol. Using free enzyme, degradation process occurs faster, resulting in a degradation of 97% after 1 hour. For the immobilized enzyme, the process is slower, presenting 87% of phenol degradation after 4 hours of reaction. However, it was found that it is possible to reuse the enzyme-support complex obtaining a percentage of 70% of phenol degradation after reuse. Hereupon, the possibility of reutilization brings enormous economical and environmental advantages.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

Abbreviations

ABTS	2.2'-azino-bis(3-ethylbenzathiazoline-6-sulfonic) acid
ADH	Alcohol dehydrogenase
AOX	Organic halide compounds
APTS	(3-Aminopropyl)triethoxysilane
ATR	Attenuated Total Reflectance ZnSe crystal plate
BET	Brunauer-Emmett-Teller
CNT	Carbon nanotube
COD	Chemical oxygen demand
Cys	Cysteine
DMP	Dimethoxy phenol
DWNT	Double-wall carbon nanotubes DWNT
EDC	N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride
EPR	Electronic paramagnetic resonance
FET	Field-effect transistor
FTIR	Fourier transform infrared
GLU	Glutaraldehyde
GOx	Enzymes glucose oxidase
His	Histidine
HPLC	High performance liquid chromatography
LBL	Layer-by-layer
LMS	Laccase-mediator systems
MW	Molecular weight
MWCNT	Multi-walled carbon nanotube
NHS	N-hydroxysuccinimide
PAHs	Polycyclic aromatic hydrocarbons
SWCNT	Single-walled carbon nanotube
TEM	Transmission electron microscopes
TNT	Trinitrotoluene

Variables

S_{BET}	Specific surface area	$m^2.g^{-1}$
K_M	Michaelis-Menten constant	mM
Abs	Absorbance	m^2
k	Thermal inactivation rate constant	h^{-1}
$t_{1/2}$	Half-life time	h
α	Ratio of specific activity	
ε	Molar extinction coefficient	$M^{-1}.cm^{-1}$
E	Redox potential	V (volt)

1. INTRODUCTION

1.1. Background and Project Presentation

Nowadays the term nanotechnology is being very disseminated and is very present in the scientific community. Nanotechnology describes the science and technology related to the control and manipulation of matter and devices on a scale less than 100 nm. It involves multidisciplinary fields such as physics, materials science, chemistry, biology, surface science, robotics, chemical, electrical, biomedical and biological engineering (Ahmed, Jackson et al. 2010). Surface-area effects become very important as the length scales of materials decrease. One effect of the small size of the materials is the increasing percentage of atoms that are situated on their surface. There is a famous quote from Wolfgang Pauli that was expressed long ago, saying:

"*God made the bulk, the surface was invented by the Devil*". (Adams and Barbante 2013)

With this logic, in nano-sized materials the Devil's realm has been extended enormously. In nanoscience and technology, surface physics and chemistry start dominating the material's properties and this must be duly taken into account. The large percentage of atoms on the surface for small entities, and the reactivity that this gives rise to, concerns one of the principal factors that differentiate properties of nanostructures from those of the bulk material (Adams and Barbante 2013). The big challenge of this area is to make every dimension as small as possible, as in nanoelectronics, but other times the aim is to make at least one dimension as large as possible, as in carbon nanotubes.

Carbon nanotubes (CNTs) are one example of the application of nanothecnology. The unique structure and properties (mechanical, optical, magnetic, electronic, thermal and biocompatibility) of CNTs have been intensively studied in different advanced areas of application such as electronics, energy storage, biotechnology and environmental engineering (Xie, Mai et al. 2005). CNTs have been used in biocatalysis mainly as carriers for enzyme immobilization (Feng and Ji 2011, Saifuddin, Raziah et al. 2013, Silva, Tavares et al. 2014). Compared to classical carriers, such as silica and polymeric

materials, CNTs present some interesting advantages as large specific surface area, high adsorption capacity, high conductivity and high enzyme loading capacity. In general, free enzymes are less stable and can be deactivated by extreme conditions of pH, temperature and organic solvents. Immobilization of enzymes on solid carriers, and particularly on CNTs, is an interesting technique for improving the catalytic performance and stability, selectivity and reusability. Different techniques for enzyme immobilization on CNTs, including adsorption, covalent bonding, entrapment or encapsulation have been described in the literature (Asuri, Karajanagi et al. 2006, Gao and Kyratzis 2008, Anne De, Alexandre et al. 2013). Noncovalent and covalent conjugations have been reported for the immobilization of various enzymes (Gao and Kyratzis 2008). Noncovalent attachment preserves the unique properties of both enzymes and CNTs, but the immobilized protein can be gradually lost during the use of the CNT-enzyme complex (Gao and Kyratzis 2008). Covalent conjugation provides durable attachment, but the enzyme structure may be more disrupted. Functionalization of CNTs with organic, polymeric, and biological molecules can provide biocompatible CNT composites with specific groups on their surface. CNT composites can provide a basis for specific immobilization of an enzyme. No matter what method is used, the main challenge is promoting the stable attachment of enzymes while maintaining their activity and function as closely as possible to their native state (Pedrosa, Paliwal et al. 2010).

Laccases (EC 1.10.3.2, p-diphenol oxidase) are attractive and industrially relevant enzymes belonging to the family of multi copper oxidases. Laccases have the capability of catalyzing the 4-electron reduction of oxygen to water with concomitant oxidation of a broad range of substrates. This enzyme can be used in various biocatalytic processes such as pulp and paper delignification, bioremediation including waste detoxification, or textile dye removal, among others (Polak and Jarosz-Wilkolazka 2012, Tamayo-Ramos, van Berkel et al. 2012, Nair, Demarche et al. 2013). The covalent immobilization of laccase can be an advantage for industrial applications, mainly in what concerns its reutilization. Thus it is very important to have a deep knowledge about the activity of this enzyme when immobilized on CNTs under different operation conditions in order to evaluate its potential use in different applications.

1.2. Main Objectives

The use of different supports for enzyme immobilization has been studied over the years. One of the most effective carriers for the immobilization of enzymes, in particular laccase, are CNTs. Laccase is an enzyme with several applications at industrial level and its immobilization brings huge economical and environmental advantages. The aim of this work is to evaluate the behavior of laccase immobilized on carbon nanotubes for biocatalysis applications. Another goal is to evaluate the influence of different CNT's surface modification techniques on the efficiency and stability of the obtained CNT-laccase bioconjugates. Specific objectives include: functionalization and characterization of carbon nanotubes by different techniques; enzyme immobilization over CTNs in order to increase its stability and reusability; evaluation of the biocatalytic ability of free and immobilized enzyme; reusability of the carrier-enzyme complex in biocatalytic processes.

1.3. Thesis organization

This thesis is divided into 7 chapters:

Chapter 1 describes the main objectives of this work, background and motivations for its developments. It serves as a guideline to the overall work presented in the further chapters.

In Chapter 2 a review of the literature is provided, situating the work within the context of existing published reports. This chapter is divided into four parts: in the first one, it is presented the enzyme used in this work, laccase, its properties and applications. In the second part, a general overview of enzyme immobilization is made. Their advantages and disadvantages and researches that have already been performed are described. In the third part, the main features of carbon nanotubes are described. The last part is related to immobilization of enzymes on carbon nanotubes, where a theoretical introduction to this theme is made describing the different techniques used for enzyme immobilization on this type of materials.

Chapter 3 refers to materials and methods used in the experimental work presented in this thesis. The first part focuses on the functionalization of the MWCNTs. The second

part presents the methodology used for the immobilization of laccase and for all the tests associated with MWCNT-laccase conjugates.

In chapter 4, results are presented and discussed, following the same pattern of the previous chapter. Firstly, the changes on the surface of carbon nanotubes caused by the different functionalization methods are discussed and correlated to the immobilization yield and recovered activity. After that, thermal stability and reutilization tests were performed in order to find the best functionalization approach. Finally, the degradation of phenol was performed using laccase immobilized in the most promising support. Results were compared with those obtained for free laccase.

Finally, chapter 5 gives the conclusions of the work presented, chapter 6 gives some proposals for future research and a final appreciation of the entire work, and the references are listed in Chapter 7.

2. LITERATURE REVIEW

2.1. Laccase: Properties and applications

Laccases (Fig. 1) are enzymes that belong to the interesting group of multi copper oxidases. They have received much attention of researchers in the last decades due to their ability to oxidize both phenolic and non-phenolic compounds as well as highly recalcitrant environmental pollutants. This enzyme is responsible for the oxidation of a broad range of organic substrates (Piontek, Antorini et al. 2002). The ability to oxidize makes these enzymes very useful for their application in several biotechnological processes. Laccase is widely distributed in higher plants and fungi (Messerschmidt and Huber 1990) and has been also found in insects and bacteria. Because of their wide reaction capabilities as well as the broad substrate specificity, laccase possess great biotechnological potential.

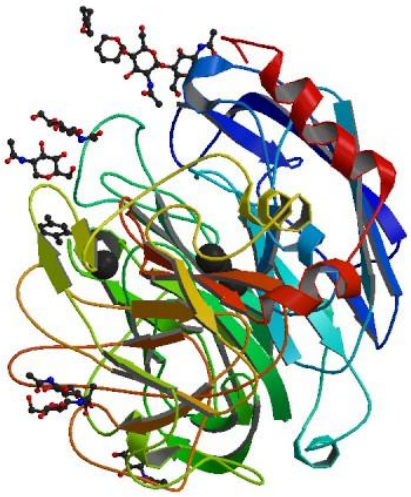

Fig. 1 - Active Laccase from *Trametes versicolor*
(source: http://www.rcsb.org/)

2.1.1. Properties of laccases

Currently more than 100 laccases have been discovered purified and partially characterized. The physical and biochemical properties of laccase rely heavily on the species from which it is isolated (Table 1). The optimum pH for fungal laccases activity is situated in the acidic pH range. Fungal laccases have a pH optimum between pH 3.6 and 5.2, while laccase from *Rhus vernicifera* like plants have pH optimum between 6.8 and 7.4. A highest activity at pH of 2-3 was observed for the oxidation of non-phenolic substrates, such as ABTS (Xu, Shin et al. 1996, Garzillo, Colao et al. 2001). Phenolic compounds like dimethoxy phenol (DMP), guaiacol and syringaldazine exhibit higher values between 4.0 and 7.0 (Garzillo, Colao et al. 2001). The isoelectric point of fungal laccase is in the pH range of 3-7.

The optimum temperature that maximizes laccase activity usually depends on its source. In general, laccases have optimum operation temperatures at 30-50°C and rapidly lose activity at temperatures above 60°C (Galhaup, Goller et al. 2002, Palonen, Saloheimo et al. 2003, Junghanns, Moeder et al. 2005). Nevertheless, the thermal stability also depends on the type of laccase. For example, the half-life at 50°C ranges from minutes in *B. cinnerea* (Slomczynski et al., 1995), to over 2–3 h in *Lentinula edodes* and *A. bisporus* (D'Annibale et al., 1996), to up to 50–70 h in *Trametes sp.* (Babu et al., 2012).

Table 1 - Physical and biochemical properties of laccase (Babu et al., 2012).

Species from which Laccase was isolated	**Substrate**	**MW (kDa)**	**(pI)**	**K_M (µM)**	**pH**	**T (°C)**	**Reference**
Coprinus cinereus	ABTS	58	4.0	26	4.0	60-70	Schneider *et al.*(1999)
Lentinula edodes Lcc1	ABTS	72	3.0	108	4.0	40	Nagai *et al.* (2002)
Marasmius quercophilus	ABTS	65		8	2.6	80	Farnet *et al.* (2004)
Melanocarpus albomyces	ABTS	80	4.0		3.5	65	Kiiskinen *et al.* (2002)
Pleurotus Ostreatus POXA 3b	ABTS	83-85	4.3	74	3.6	35	Palmieri *et al.* (2003)

Trametes gallica Lac 1	ABTS	60	3.1	12	2.2	70	Dong & Zhang (2004)
Volvariella volvacea	ABTS	58	3.7	30	3.0	45	Chen *et al.* (2004)
Botrytis cinerea	DMP	74	4.0	100	3.5	57	Slomczynski *et al.* (1995)
Coniothyrium minitans	DMP	74	4.0	100	3.5	60	Dahiya *Etal.*(1998)
***Trametes gallica Lac* II**	DMP	60	3.0	410	3.0	70	Dong & Zhang (2004)
Pleurotus ostreatus POXA3a	DMP	83-85	4.1	14000	5.5	35	Palmieri *et al.*(2003)
Pleurotus ostreatus POXA2	DMP	67	4.0	740	6.5	25-35	Palmieri *et al.*(1997)
Cerrena maxima	Guaiacol	57-67	3.5	160 - 300		50	Shleev *et al.*(2004)
Pleurotus ostreatus POXC	Guaiacol	59	2.9	1200	6.0	50-60	Palmieri *et al.*(1997)
Thelephora terrestris	Guaiacol	66		121	4.8	45	Kanunfre & Zancan (1998)
Daedalea quercina	Syringaldazine	69	3.0	131	7.0	55	Baldrain (2004)
Magnaporthe grisea	Syringaldazine	70		118	6.0	30	Iyer & Chattoo (2003)
Marasmius quercophilus	Syringaldazine	60		4.2	4.5	80	Farnet *et al.(* 2004)
Pleurotus ostreatus POXA1w	Syringaldazine	61	6.7	130	6.0	45-65	Palmieri *et al.*(1997)

Regarding laccase structure, it is considered an enzyme with a good stability due to the high level of glycosylation (Durán, Rosa et al. 2002). The laccase molecule, as an active holoenzyme form, is a dimeric or tetrameric glycoprotein, usually containing (per monomer) four copper (Cu) atoms bound to three redox sites (Type 1, Type 2 and Type

3 Cu pair). For having catalytic activity a minimum of four Cu atoms per active protein unit is needed. Three types of copper can be distinguished using UV/visible and electronic paramagnetic resonance (EPR) spectroscopy. Type 1 Cu (T1) at its oxidized resting state is responsible for the blue color of the protein at an absorbance of approximately 610 nm and is EPR detectable. Type 2 Cu (T2) does not confer color but is EPR detectable. Type 3 Cu (T3) atoms consists of a pair of Cu atoms in a binuclear conformation that gives a weak absorbance in the near UV region but no detectable EPR signal. The Type 2 and Type 3 copper sites are close together and form a trinuclear center that are involved in the catalytic mechanism of the enzyme (Fig. 2) (Thurston 1994).

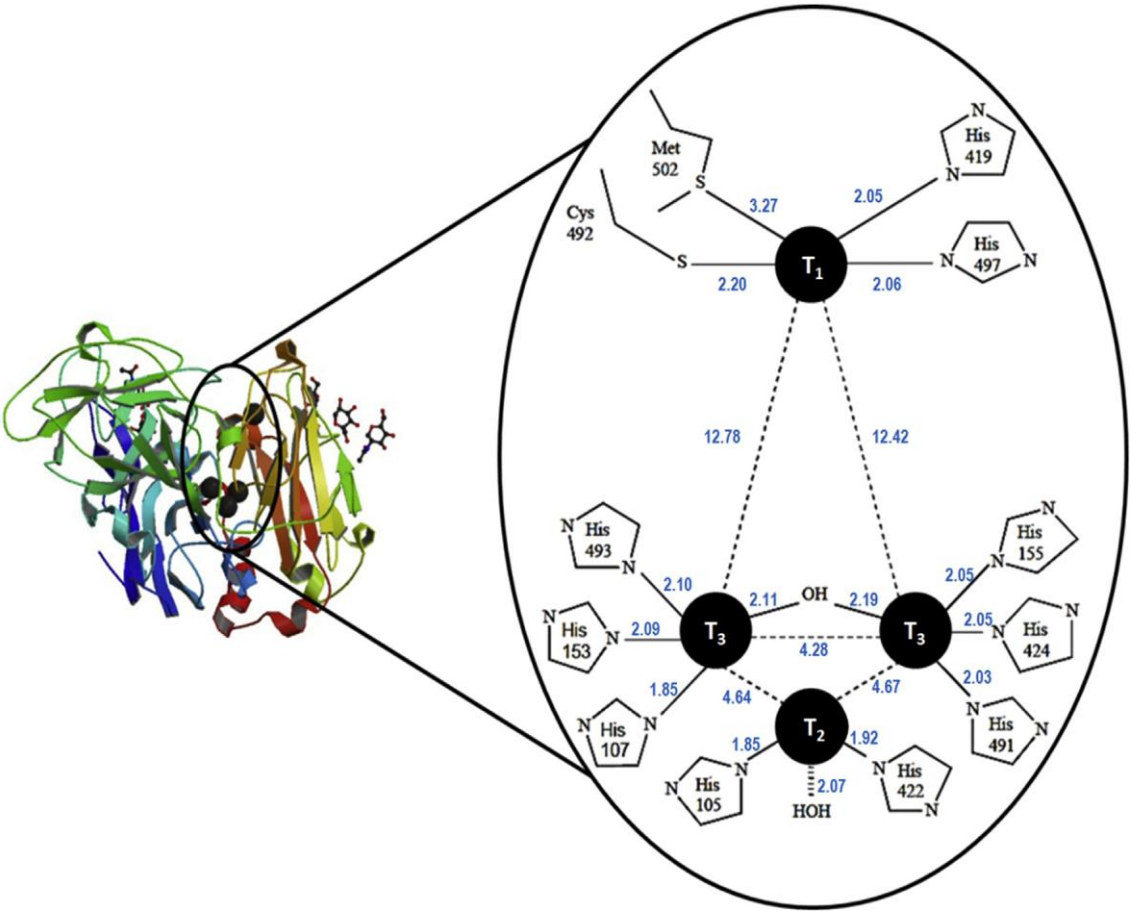

Fig. 2 - Laccase active site (Rivera-Hoyos, Morales-Álvarez et al. 2013)..

Three main steps are involved in the mechanism of laccase action. Initially, Cu T1 is reduced by the action of a reducer substrate, which is subsequently oxidized. Then the electron is internally transferred from Cu T1 over >13 Å through a Cys-His pathway to the trinuclear center, formed by the atoms of coppers T2 and T3. The oxygen molecule binds to the trinuclear center for an asymmetric activation through a substrate binding site located near the His ligands of Cu T1 center. It has been proposed that the joining pocket for O_2 seems to restrict the access of oxidizing agents different than O_2. During the steady state of the process H_2O_2 is not detected outside the enzyme, indicating that reduction of four electrons from the O_2 to H_2O is occurring (Rivera-Hoyos, Morales-Álvarez et al. 2013). Since oxidation of an electron in the phenolic substrate is linked to reduction of four oxygen electrons, it cannot be assumed the reaction's mechanism is simple. Therefore, it is assumed that laccase acts as a battery, storing electrons from individual oxidation reactions in order to reduce molecular oxygen. Therefore, oxidation of four substrate molecules is required to produce complete reduction of molecular oxygen to water (Solomon, Augustine et al. 2008).

During laccase's catalytic process, different free radical reactions result, depending on structure and reaction conditions. The most frequent reactions are coupling of free radicals that generate dimeric products or polymeric compounds and oxidative carboxylations. The oxidation of substrates is coupled to reduction of molecular oxygen, generating two water molecules. For each oxygen reduced, four molecules of substrate are oxidized without hydrogen peroxide production (Solomon, Augustine et al. 2008).:

$$4H^+ + 4\ \text{substrate} + O_2 \rightarrow 2H_2O + 4\ \text{substrate}^+$$

Consequently, laccases are considered "ideal green" catalysts because they employ O_2 as a co-substrate and generate H_2O as a byproduct (Fig. 3).

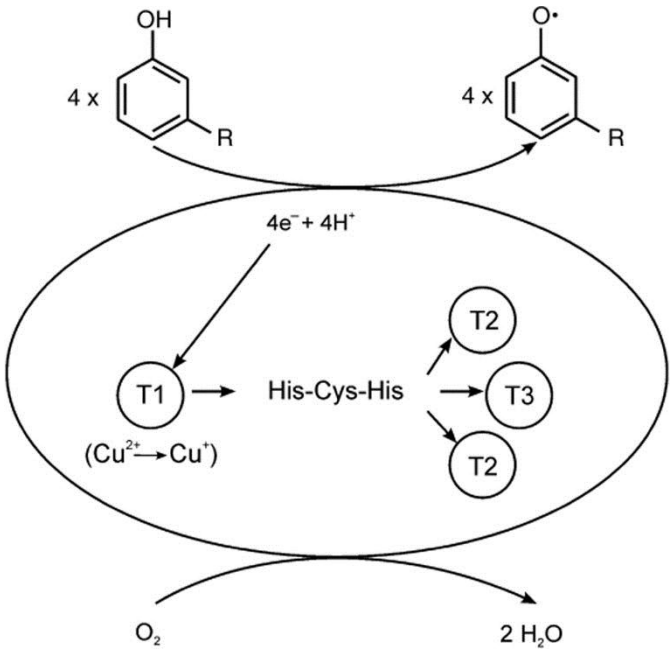

Fig. 3 - Laccase catalytic cycle (Baldrian 2006).

Laccase can also be used as a mediator system with some chemical mediators. Due to the large dimension of the laccase molecule (Rodgers, Blanford et al. 2010) and due to its low redox potential (≤0.8 V) it is often necessary to use other compounds forming a mediator system. As it can been seen in figure 4 there are two mechanisms by which compounds can be oxidized under the catalytic action of the enzyme. In typical interactions of laccase with a substrate, the catalytic site of the enzyme abstracts electrons from the substrate and releases an oxidized product. In cases where a mediator is present, the mediator can be oxidized by the enzyme and subsequently oxidize another compound that is either a substrate or non-substrate resulting in the formation of oxidized product(s) and regeneration of the mediator (Banci, Ciofi-Baffoni et al. 1999). These mediators are low molecular weight compounds that can easily be oxidized by laccase, producing very reactive and unstable cationic radicals. However, at the same time these cationic radicals can oxidize complex compounds (not including phenolic substrates) before returning to their original state (Torres, Bustos-Jaimes et al. 2003). By this mechanism mediators act as diffusible electron transporters, allowing indirect oxidation of polymeric substrates such as lignin, penetrating even to less accessible areas of its structure. Additionally, because of mediator use, laccases are able to oxidize

compounds with greater redox potential than their own; an example of this is the oxidation mediated by polycyclic aromatic hydrocarbons or PAHs (Riva 2006). Since Bourbonnais demonstrated that mediator inclusion expanded laccase's catalytic activity toward non-phenolic substrates (Bourbonnais and Paice 1990), more than 100 different mediators have been described with ABTS (2.2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and HBT (1-hydroxybenzotriazole) being the most commonly used. This laccase-mediator system can be applied in diverse oxidative reactions (Rivera-Hoyos, Morales-Álvarez et al. 2013).

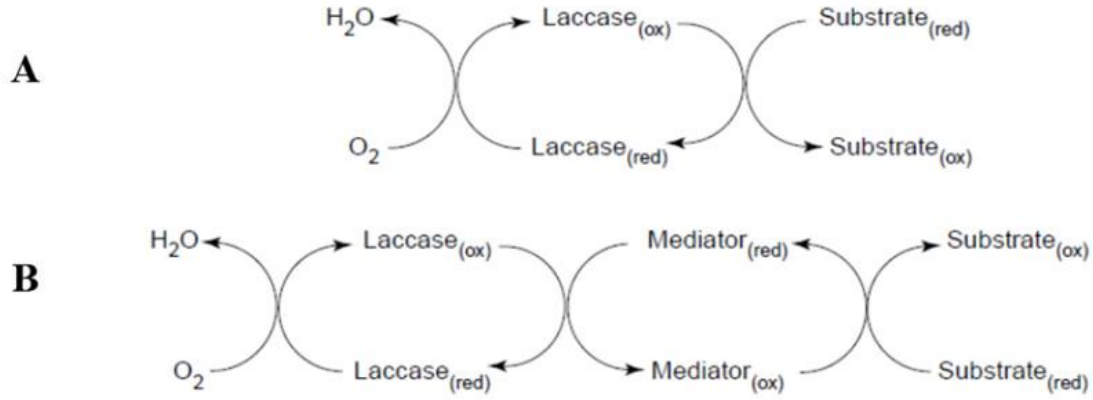

Fig. 4 - Schematic representation of laccase catalyzed redox cycles for subtrate oxidation in the absence (A) or in the presents (B) of redox mediators (Riva 2006).

2.1.2. Biotechnological applications

Laccases are of great interest to industry, and have been used in many processes such as delignification of lignocellulosic compounds, biopulping and biobleaching, transformation of colorants in the textile industry, wastewater treatment and degradation of explosives and pesticides (Singh Arora and Kumar Sharma 2010, Shraddha, Shekher et al. 2011).

The laccases are widely used in the paper industry. Laccase from *T. versicolor* was capable of paper pulp delignification in the presence of mediators, without the need of traditional chlorinated toxic reagent techniques (Bourbonnais, Paice et al. 1997). Many studies have been developed to optimize the entire process (Ferraroni, Matera et al. 2012). Due to its high redox potential laccase can be used in almost all paper product

production chain: paper pulp development, pulp chlorine-free washing, or for effluent treatment. It is of common knowledge that pollution load in waste-water resulting from the washing process during paper production, is characterized by high levels of chemical oxygen demand (COD), color, and more than 500 different absorbable organic halide compounds (AOX), (Savant, Abdul-Rahman et al. 2006). In relation to forest product industry there are two areas that have been investigated recently: i) design of lignocellulosic materials with new resistance and stability properties by means of phenolic compound grafts catalyzed by laccase, in the so-called "functionalization of cellulose fibers"; ii) laccase use for improving compression degree in woodbased panels (through "in situ" enzyme lignin coupling), without the use of toxic adhesives containing formaldehyde (Rivera-Hoyos, Morales-Álvarez et al. 2013).

Laccase's application in the textile industry range from cotton fiber washing, textile dye and bleaching as well as precursor of coloring matter production to elimination of the former in waste-waters (Rivera-Hoyos, Morales-Álvarez et al. 2013). For example, laccase can be utilized for bioremediation in matrices containing compounds such as PAHs, chlorophenols, dimethoxyphenols, nitrophenols, and pesticides, askarels, among others (Gayosso-Canales, Rodríguez-Vázquez et al. 2012). In fact, laccase from *M. thermophila* has been recently used for indigo dye oxidation in denim cloth (Riva 2006).

Degradation of PAHs is considered of particular interest, since polycyclic aromatic hydrocarbons are a group of very dangerous xenobiotics (mutagenic, carcinogenic and/or teratogenic) widely distributed in terrestrial and aquatic environments. The main emission sources come from marine dumping, vehicle engines, industrial processes and forest fires. Recent trends for PAHs elimination aim to combine chemical and biological methods, such as shock treatment for oxidation (Riva 2006).Thus, a greater effort is being made to design effective PAHs oxidation by laccases. These procedures require demanding process conditions, such as the presence of organic solvents and extreme environmental conditions, amongst others (Kudanga, Nyanhongo et al. 2011).

The laccases also have characteristics which enables them to interact with the area of nanobiotechnology. This enzyme can be used in the development of biosensors for clinical and environmental analysis and be employed in the advancement of biofuel for clean electrical energy (without fossil fuel use) through laccase immobilization in the

cathode. For example, laccase's enzymatic activity coupled to physical transducers can be useful in biosensor design to detect O_2 and a wide variety of substrate reducers as phenols, anilines and glucose. In addition, indirect activity of other enzymes (for example, amylases, aminopeptidases, alkaline phosphatase, cellobiose oxidase, chymotrypsin or glucosidase) can be determined (Rivera-Hoyos, Morales-Álvarez et al. 2013). For the pharmaceutical industry laccases have been used for the development of antitumor agents, new antibiotic derivatives and cosmetics.

Finally, laccases can catalyze the reduction of oxygen to water, a promising laccase application is aimed at biofuel design and cleaning-up of certain explosives in soil, such as trinitrotoluene (TNT), (Shraddha, Shekher et al. 2011).

In sum, laccases are enzymes with a high potential in various areas of the industry. They are very promising "green tools" in various processes, attracting a high attention from the scientific community.

2.2. Immobilization of Enzymes

Enzymes, including laccase are able to catalyze reactions in three different states: as free molecules in solution, in aggregates with other entities, and attached to surfaces.

The attached or immobilized state has been of particular interest to those wishing to exploit enzymes for technical purposes. Enzyme immobilization has brought, in most cases, improvements at economic level and in the performance of industrial processes (Table 2).

Table 2 - Technological properties of immobilized enzyme systems (Brena and Batista-Viera 2006).

Technological Properties of Immobilized Enzyme Systems	
Advantages	***Disadvantages***
Catalyst reuse	Loss or reduction in activity
Easier reactor operation	Diffusional limitation
Easier product separation	Additional cost
Wider choice of reactor	

The first industrial use of immobilized enzymes was reported in 1967 by Chibata and co-workers, who developed the immobilization of *Aspergillus oryzae* aminoacylase for the resolution of synthetic racemic D-L amino acids (Tosa and Shibatani 1995).

There are several applications for the enzyme immobilization process, for example, the industrial production of sugars, amino acids, and pharmaceuticals (Tosa and Shibatani 1995) (Table 3).

Aside from the application in industrial processes, the immobilization techniques are the basis for making a number of biotechnological products with applications in diagnostics, bioaffinity chromatography, and biosensors (Brena and Batista-Viera 2006). Therapeutic applications are also foreseen, such as the use of enzymes in extra–corporeal shunts (Chang 1991).

In the past decades, immobilization technology has developed rapidly and has increasingly become a matter of rational design; but there is still the need for further development (Brena and Batista-Viera 2006). Extension of the use of immobilized

enzymes to other practical processes will require new methodologies and a better understanding of current techniques.

Table 3 - Major products obtained using immobilized enzymes (Anne De, Alexandre et al. 2013).

Major Products Obtained Using Immobilized Enzymes	
Enzyme	**Product**
Glucose isomerase	High-fructose corn syrup
Amino acid acylase	Amino acid production
β-Galactosidase	Semi-synthetic penicillins
Penicillin acylase	Acrylamide
Nitrile hydratase	Hydrolyzed lactose (whey)

Conceptually, there are two basic methods for enzyme immobilization, as the enzyme-support link may take place by physical or chemical interactions. These different types of links involve several types of immobilization methodologies. Physical coupling methods include the entrapment of the enzyme in a tridimensional matrix or its encapsulation in an organic or inorganic polymer (membranes) (Matijošytė, Arends et al. 2010), whereas chemical coupling can occur through adsorption, covalent binding to the carrier or self-immobilization (no support required).These techniques are reviewed by (Sheldon 2007). Each of these methodologies presents advantages and disadvantages.

Entrapment is defined as the physical retention of enzymes in a porous solid matrix, such as polyacrylamide, collagen, alginate or gelatin (Lu, Zhao et al. 2007, Niladevi and Prema 2008). The enzyme is first suspended in the monomer solution, and a subsequent polymerization process keeps the enzyme trapped, preventing direct contact with the environment (Fig. 5(a)). Similar behavior is observed in the encapsulation of enzymes in comparison to the entrapment method, because the enzyme is protected from the environment and mass transfer represents a serious limitation in both of these immobilization methods (Brady and Jordaan 2009) (Fig. 5(b)).

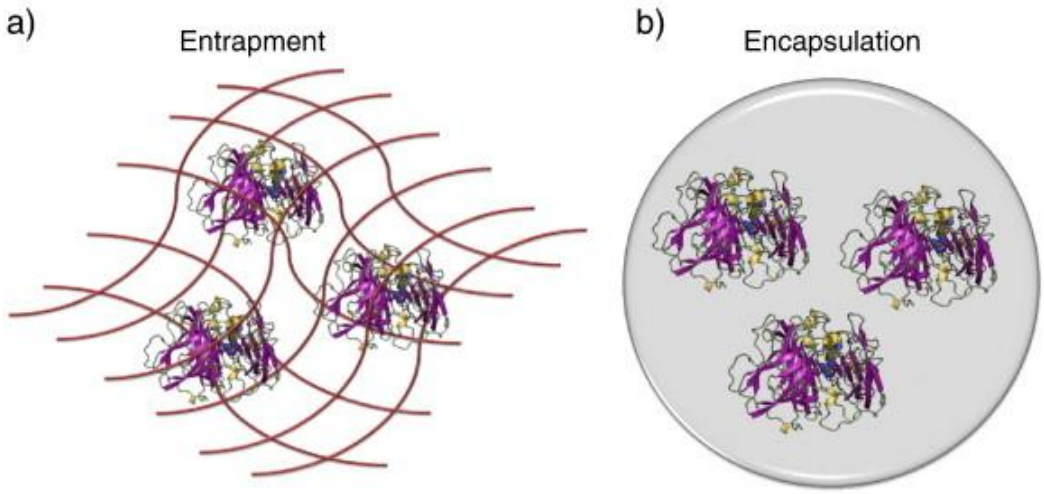

Fig. 5 - Immobilization of enzymes by physical interactions. (a) Entrapment of enzymes into a porous solid matrix. (b) Encapsulation of enzymes (Fernández-Fernández, Sanromán et al. 2013).

The adsorption method onto a support is based on ionic and/or other weak forces of attraction (Fig. 6(a)). Adsorption is a relatively simple and inexpensive method for laccase immobilization and may therefore have a higher commercial potential than other methodologies (Bayramoğlu and Yakup Arıca 2009, Brady and Jordaan 2009). Regarding to covalent binding, chemical groups on the support surface are activated and react with nucleophilic groups on the protein (Arroyo, 1998) (Fig. 6(b)). Most enzymes are covalently attached using their lysine amino groups because of their frequent presence on the protein surface and high reactivity (Brady and Jordaan 2009).Finally, the use of solid supports for enzyme immobilization may reduce the specific and volumetric activity of the biocatalyst. Therefore, self-immobilization is possible with the use of bifunctional cross-linkers (Brady and Jordaan 2009) (Fig. 6(c)) . These cross-linkers include dialdehydes, diiminoesthers, diisocyanates and diamines activated by carbodiimide (Arroyo, 1998).

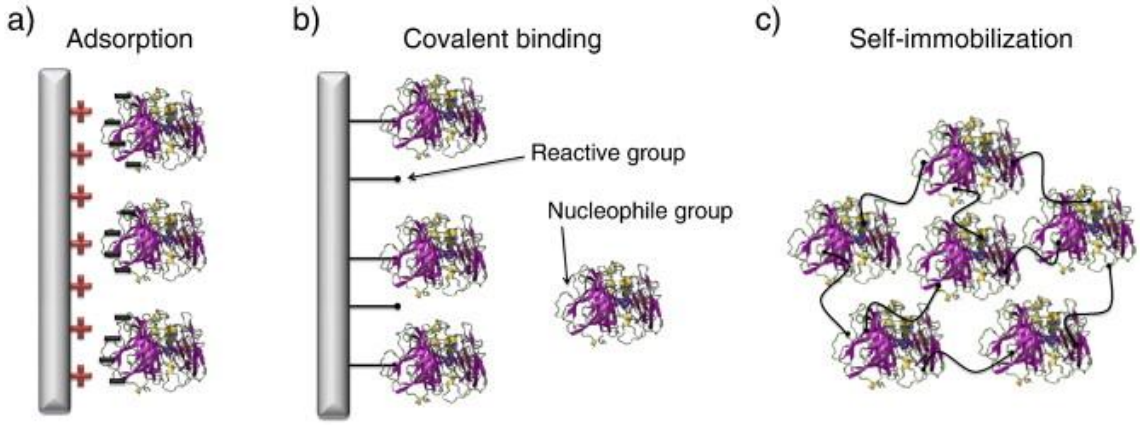

Fig. 6 - Chemical interactions for enzyme immobilization. (a) Adsorption of enzymes onto a support by ionic forces. (b) Covalent binding between the nucleophilic groups of the enzyme and the support. (c) Self-immobilization: model of cross-linked enzyme aggregates (CLEAs) (Fernández-Fernández, Sanromán et al. 2013).

Regarding to laccase, some potential applications using different immobilization methods are summarized in Table 4.

Table 4 - Recent methods for laccase immobilization (Fernández-Fernández, Sanromán et al. 2013).

Laccase source Entrapment	Support	Substrate	Application	Reference
Trametes versicolor	Sol–gel matrix of diglycerysilane	Phenols	Biosensor	(Montereali et al., 2010)
Lentinus polychrous	Cu, Zn and Ca–alginate beads	ABTS	Dyes decolorization	(Phetsom et al., 2009)
Trametes versicolor	Hydrogel structures and semi-IPNs.	ABTS	Dyes decolorization	(Yamak et al., 2009)

Myceliophthora thermophila	Alginate/gelatin with PEG		Dyes decolorization	(Wang et al., 2008b)
Encapsulation				
Trametes versicolor	Alumina pellets with self-assembled LbL	ABTS	Paper industry	(Crestini et al., 2010)
Unspecified	Sol–gel silica	ABTS	Xenobiotics degradation	(Qiu and Huang, 2010)
Trametes versicolor	Poly(ethyleneimine) (PEI) microcapsules			(Zhang and Rochefort, 2010)
Adsorption				
Trametes hirsuta	Graphite electrodes	Catechol	Biosensor	(Shleev et al., 2006
Pycnoporus sanguineus	Magnetic chitosan microspheres	ABTS		(Jiang et al., 2005a and Jiang et al., 2005b)
Trametes versicolor	Carbon based electrodes. Carbon nanotubes	ABTS	Biofuel cell	(Rubenwolf et al., 2010)

Trametes versicolor	Mesoporous silica: SBA-15		Xenobiotics degradation	(Fernando Bautista et al., 2010)
Covalent binding				
Coriolus versicolor	Chitosan		Xenobiotics degradation	(Zhang et al., 2008)
Pleurotus sajor-caju	Polyamide 6.6 membranes	ABTS	Dyes decolorization	(Rekuć et al., 2009a)
Trametes versicolor	Polypropylene membranes	ABTS	Biofuel cell	(Georgieva et al., 2010)
Self-immobilization				
Coriolopsis polyzona	CLEAs with polyethylene glycol and GLU	ABTS	Xenobiotics degradation	(Cabana et al., 2007)
Myceliophthora thermophila	Spherezymes. Cross-linked with GLU and ethylenediamin	ABTS		(Jordaan et al., 2009)

2.3. Carbon Nanotubes (CNTs)

A Carbon Nanotube is a tube-shaped material, made of carbon, having a diameter measuring on the nanometer scale. The carbon nanotubes are formed essentially by a sheet of graphite having various structures nevertheless differ from each other by the length, thickness, number of layers and the type of helicity. The characteristics depend on these variations, and they can act as metals or semiconductors. In the present study, carbon nanotubes will act as support for the immobilization of the enzyme. Depending on the number of graphite layers, carbon nanotubes can be categorized as single-wall nanotubes (SWNT), double-wall nanotubes (DWNT) or multi-wall nanotubes (MWNT).

2.3.1. Single-wall Nanotubes (SWNT)

A single-wall nanotube has a simple cylindrical wall. They have graphite tubes that are normally capped at the ends and its structure is the layer of graphite, a single atom thick, called graphene, which is rolled into a seamless cylinder. Typically SWNT have a diameter close to 1 nm and the length can be thousands of times longer than diameter. SWNT are more pliable yet harder to make than MWCNT. They can be twisted, flattened, and bent into small circles or around sharp bends without breaking (Fig. 7).

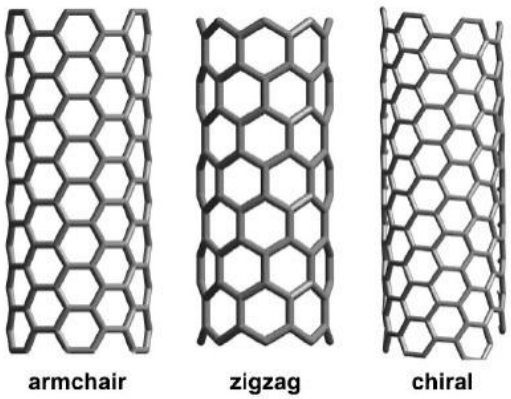

Fig. 7 - Structures of SWCNTs
(source: http://coecs.ou.edu/Brian.P.Grady/images/nanotube.jpg)

2.3.2. Double-wall Nanotubes (DWNT)

Double-wall nanotubes have morphology and properties similar to SWNT and at the same time due to their double wall have a greater chemical resistance. This property is especially important when functionality is required to add new properties to the nanotube. Because they are developed for highly specific applications, SWNT that have been functionalized are more susceptible to breakage. Creating any structural imperfections can modify their mechanical and electrical properties. However, with DWNT, only the outer wall is modified, thereby preserving the intrinsic properties. Also, research has shown that DWNT have better thermal and chemical stability than SWNT. DWNT can be applied to gas sensors and dielectrics, and to technically-demanding applications like field-emission displays, nanocomposite materials, and nanosensors.

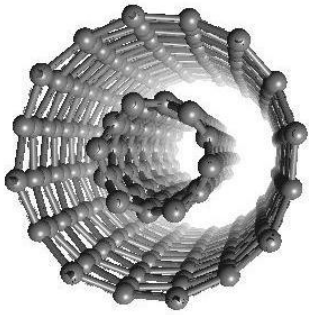

Fig. 8 - Straight double-walled Carbon nanotube (Source: http//www.asu.edu/clas/css/NUE/gallery.html)

2.3.3. Multi-wall Nanotubes (MWNT)

Multi-wall nanotubes differ from single-wall nanotubes, for example, in diameter, the diameter of multi-wall nanotubes being between 5 nm and 50 nm. The interlayer distance in MWNT is close to the distance between graphene layers in graphite.

This type of nanotubes may appear either in the form of a coaxial assembly of SWNT similar to a coaxial cable, or as a single sheet of graphite rolled into the shape of a scroll.

Due to its structure is easier to produce them in large volumes. However, they are more susceptible to the occurrence of imperfections. Regions of structural imperfection may diminish its desirable materials properties.

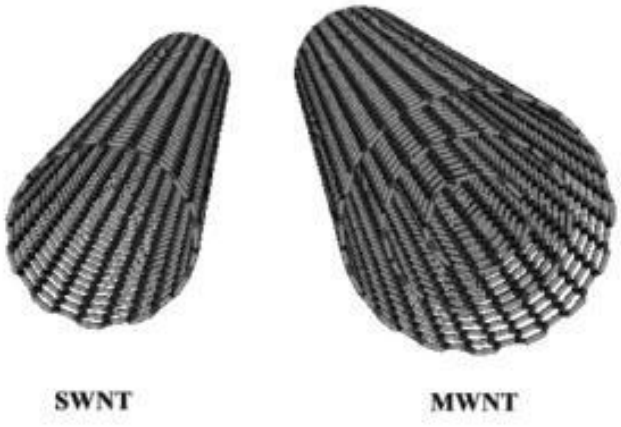

Fig. 9 - Single-walled carbon nanotube and multi-walled carbon nanotube (Source: itech.dickinson.edu)

The prices of these nanotubes are cheaper than the above, precisely because of its production be easier.

2.3.4. Properties of a Carbon Nanotube

The intrinsic mechanical and transport properties of carbon nanotubes make them the ultimate carbon fibers. Table 5 and Table 6 compare these properties to other engineering materials.

Overall, Carbon Nanotubes show a unique combination of stiffness, strength, and tenacity compared to other fiber materials which usually lack one or more of these properties. Thermal and electrical conductivity are also very high, and comparable to other conductive materials.

Table 5 - Mechanical properties of engineering fibers *(http://www.nanocyl.com/en).*

Fiber Material	Specific Density	E (TPa)	Strenght (GPa)	Strain at Break (%)
Carbon Nanotube	1.3 - 2	1	10-60	10
HS Steel	7.8	0.2	4.1	<10
Carbon Fiber-PAN	1.7 - 2	0.2-0.6	1.7-5	0.3-2.4
Carbon Fiber-Pitch	2 - 2.2	0.4-0.96	2.2-3.3	0.27-0.6
E/S - glass	2.5	0.07/0.08	2.4/4.5	4.8
Kevlar* 49	1.4	0.13	3.6-4.1	2.8

E – Young modulus

*Kevlar is a registered trademark of DuPont.

Table 6 - Transport properties of conductive materials *(http://www.nanocyl.com/en)*

Material	Thermal Conductivity (W/m.k)	Electrical Conductivity (S/m)
Carbon Nanotubes	> 3000	10^6 - 10^7
Copper	400	6 x 10^7
Carbon Fiber - Pitch	1000	2 - 8.5 x 10^6
Carbon Fiber - PAN	8 - 105	6.5 - 14 x 10^6

2.4. Enzymes immobilized on carbon nanotubes

Nanomaterials have served as supports for immobilization of enzymes and offer ideal characteristics such as high surface area, mass transfer resistance, and effective enzyme loading. Among various supports that can be used, carbon nanotubes are the nanomaterials that have most attention by the scientific community.

Enzyme immobilization is a promising biotechnological application of CNTs (Chen, Bangsaruntip et al. 2003, Gao and Kyratzis 2008, Zhang, Xing et al. 2009, Hansen, Liu et al. 2010), especially for fabrication of biosensors and biofuel cells (Barone, Baik et al. 2005, Song, Pehrsson et al. 2006, Tsai and Chiu 2007, Willner, Yan et al. 2009, Lee, Shin et al. 2010, Lee, Shin et al. 2011). CNTs offer unique advantages including enhanced electronic properties, a large edge plane to basal plane ratio, and rapid electrode kinetics (Jacobs, Peairs et al. 2010). To fully explore the potential of the enzyme-CNT complex, it is essential to find optimal methods for enzyme immobilization (Taft, Lazareck et al. 2004, Veetil and Ye 2007, Zhang, Xing et al. 2009).

Noncovalent and covalent conjugations have been reported for the immobilization of various enzymes (Gao and Kyratzis 2008). Noncovalent attachment preserves the unique properties of both enzymes and CNTs, but the immobilized protein can be gradually lost during the use of the CNT-enzyme complex (Gao and Kyratzis 2008). Covalent conjugation provides durable attachment, but the enzyme structure may be more disrupted.

2.4.1. Non-covalent enzyme immobilization

For the immobilization of enzymes on CNTs, compared to covalent methods, the noncovalent approach is considered to be a more promising technique, because it preserves the conformational structure of the immobilized enzymes (Matsuura, Saito et al. 2006, Nepal and Geckeler 2006).

Direct physical adsorption

With the direct physical adsorption method, the interacting force between the enzyme and CNT is predominantly a hydrophobic interaction (Gao and Kyratzis 2008). An enzyme includes hydrophobic regions on its exterior can interact with the wall of a CNT through hydrophobic interactions, as illustrated in Fig. 10. The π–π stacking interaction between the sidewalls of CNTs and the aromatic rings also contributes to the adsorption (Matsuura, Saito et al. 2006).

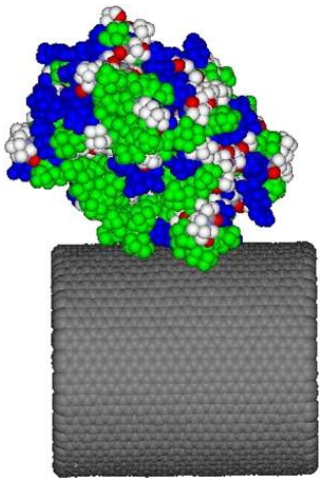

Fig. 10 - Schematic illustration of lipase adsorbed on carbon nanotubes by molecular dynamics simulation. The hydrophobic parts are in green, hydrophilic parts in blue (Feng and Ji. 2011).

Enzymes adsorbed onto CNTs functionalized with polymers

Polymers and biomolecules have been used to functionalize CNTs. The functionalized CNTs (f-CNTs) have good aqueous dispersibility, and the formation of the enzyme–CNT complex is facilitated. f-CNTs can have molecular recognition and binding specificity for enzymes (Chen, Bangsaruntip et al. 2003, Mu, Liu et al. 2008), due to the combination of many molecular properties such as hydrophobicity, electrostatic interactions, hydrogen bonding, and steric properties.

Polymers coated on CNTs can provide negatively and positively charged functional groups on the surface of CNT–polymer complexes.

For example, the surface of SWNTs can be positively charged by coating poly-(sodium 4-styrenesulfonate) combined with ionic liquids (Wu, Zhao et al. 2009), and glucose oxidase can be immobilized onto the SWNTs as illustrated in Fig. 11.

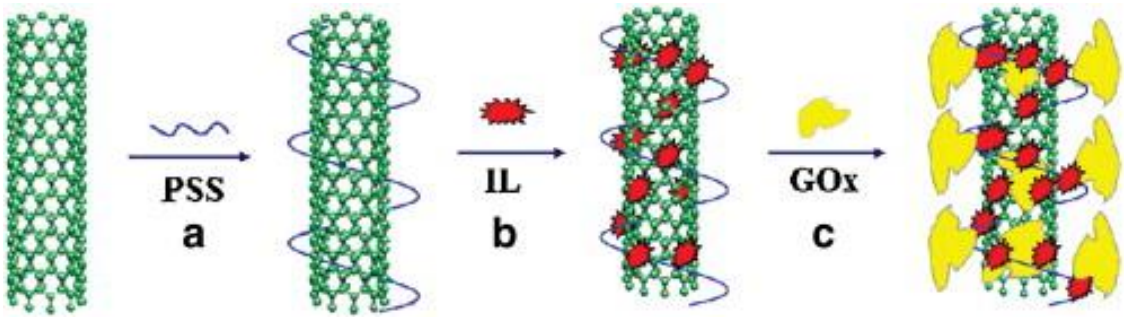

Fig. 11 - Schematic illustration of the immobilization of glucose oxidase (GOX) on the surface of SWNTs by using LBL deposition. PSS: poly (sodium 4-styrenesulfonate); IL: ionic liquid. From (Wu, Zhao et al. 2009)

Enzymes adsorbed onto the CNTs functionalized with biomolecules

Enzymes can be specifically bound to CNTs functionalized with biomolecules. Enzymes glucose oxidase (GOx) and alcohol dehydrogenase (ADH) labeled with the complementary sDNA tags can recognize their binding address and adhere to the CNTs functionalized with the complementary strands, as illustrated in Fig. 12.

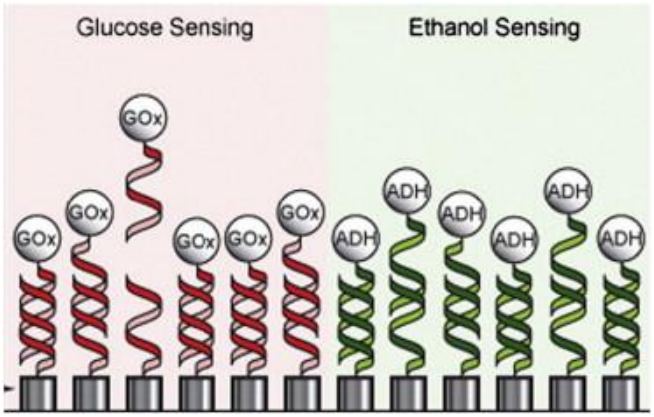

Fig. 12 - Conjugation of enzymes tagged with single-stranded DNA to the tips of designated CNTs functionalized with the complementary strands.
From *(Withey et al., 2008)*

Enzymes adsorbed onto CNTs with assistance of surfactants

Enzymes can be adsorbed onto CNTs with the assistance of surfactants. CNTs coated with the surfactant Triton X-100 can specifically bind to streptavidin as shown in Fig. 13. (Chen, Bangsaruntip et al. 2003).

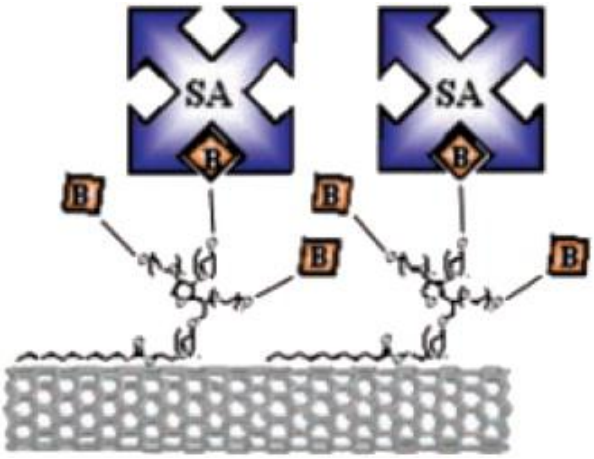

Fig. 13 - Scheme for streptavidin (SA) recognition with a nanotube coated with biotinylated Tween. From (Chen, Bangsaruntip et al. 2003)

Layer-by-layer technique for immobilization of enzymes

A layer-by-layer (LBL) approach has been adopted for immobilization of enzymes. It permits the coating of various enzymes, producing multilayer enzyme films on CNTs. The biocatalytic activity can be increased by increasing the number of enzyme layers assembled on CNTs. For example, LBL coatings were prepared by the alternating assembly of cationic lysozyme-SWNT and anionic DNA-SWNT (Nepal and Geckeler 2006) as shown in Fig. 14.

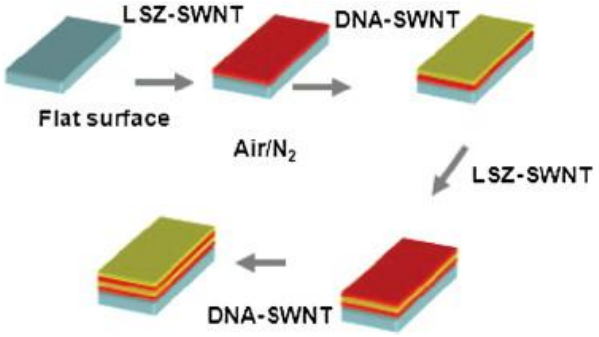

Fig. 14 - Schematic diagram of LBL assembly of LSZ-SWNT and DNA-SWNT. LSZ: lysozyme. From (Nepal et al. 2008)

2.4.2. Covalent linking

Direct covalent linking of enzymes onto CNTs

Covalent immobilization of enzymes on CNTs has been demonstrated by inducing the reaction of the free amine groups on the surface of a protein with carboxylic acid groups that are generated by sidewall oxidation of CNTs and subsequent activation using carbodiimide *(*Asuri, Karajanagi et al. 2006*)*, as presented in Fig. 15.

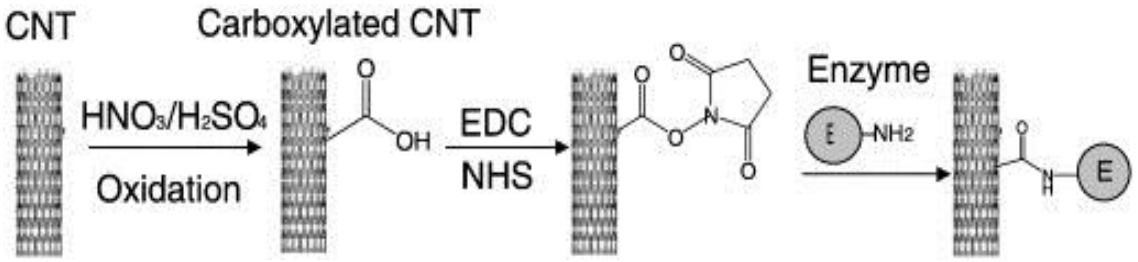

Fig. 15 - Schematic representation of conjugation of enzymes to carboxylated CNTs using EDC in the presence of NHS. EDC: N-ethyl-N-(3-(dimethylamino)propyl) carbodiimide hydrochloride; NHS: *N*-hydroxysuccinimide (Feng and Ji. 2011).

Covalent attachment of enzymes onto CNTs with linking molecules

Linking molecules can provide specific sites for CNTs to immobilize enzymes (Wang, Wei et al. 2010). The activity of perhydrolase S54V was greatly improved when immobilized on CNTs coated with the poly-(ethylene glycol) based spacer, as the spacer could prevent non-specific enzyme attachment (Dinu et al. 2010). Wang, Wei et al. 2010 cloned the annotated NADH oxidase gene from the *Bacillus cereus* genome and overexpressed the clone with pET30 vector encoding N-terminal 6× His-tag. The His-tagged NADH oxidase was then immobilized onto SWNTs functionalized with $N_{\alpha}N_{\alpha}$-bis(carboxymethyl)-l-lysine hydrate, as illustrated by Fig. 16.

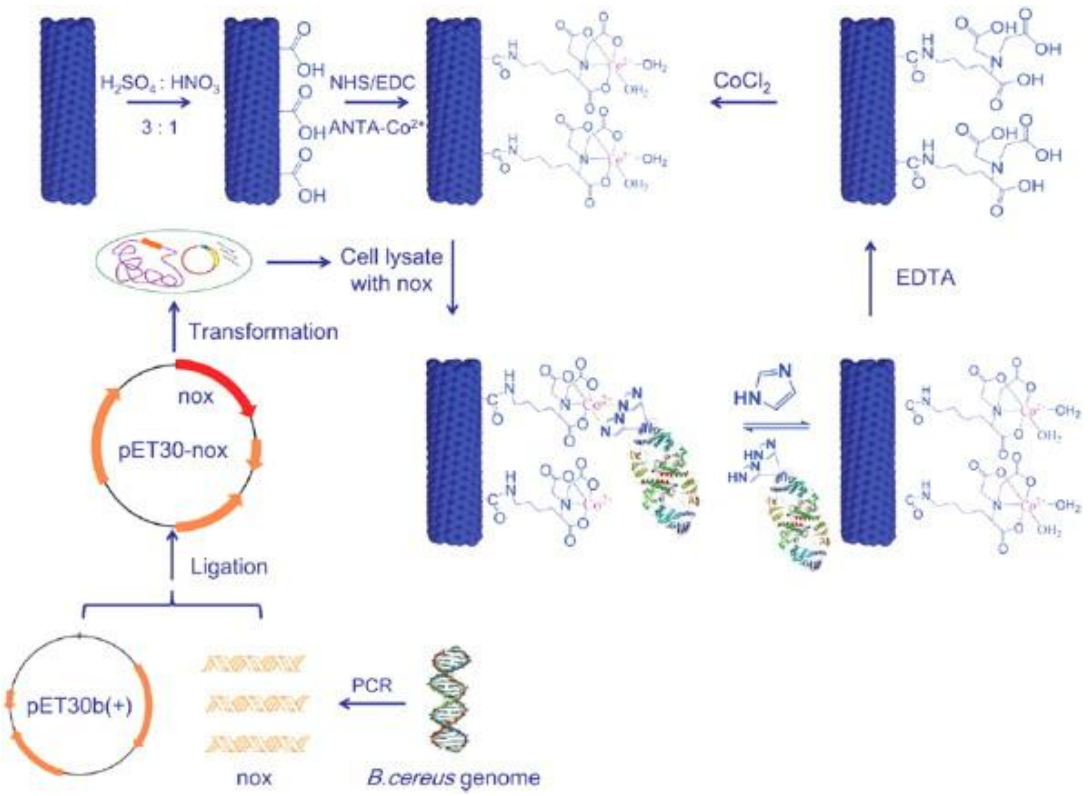

Fig. 16 - Scheme of reversible immobilization of NADH oxidase on functionalized SWNTs. From (Wang, Wei et al. 2010)

3. MATERIALS AND METHODS

3.1. Reagents

All studies were performed using commercial laccase that was kindly offered by the company Novozymes (Denmark). Commercial multi-walled carbon nanotubes (MWCNTs) with diameter range of 10-20 nm were purchase from Shenzhen Nanotechnologies Co. Ltd. (purity > 95 %, length = 5–15 μm, ash content < 0.2 wt%, amorphous carbon <3 %). 2.2'-azino-bis3-ethylbenzathiazoline-6-sulfonic acid (ABTS) was supplied by AppliChem (Germany). Glutaraldehyde, (3Aminopropyl)triethoxysilane (APTS),N-hudroxysuccinimide (NHS) and 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) were supplied by Sigma-Aldrich.

3.2. Functionalization of MWCNT

MWCNTs were firstly treated with HNO_3 at different concentrations (0.05M; 0.1M; 0.2M; 0.3M) in order to obtain oxidize their surface at different extent. Briefly, 0.2 g of MWCNTs were added to 75 mL of a HNO_3 aqueous solution with the desired concentration. The mixture was pressurized with 0.5 MPa of nitrogen and heated up to 200 ºC for 2h. The nanotubes were filtered on a glass frit, washed with water until the filtrate reached neutral pH, and dried overnight at 120 ºC. The obtained materials were labeled as MWCNTox_Y with Y being the HNO_3 concentration.

After that, the oxidized MWCNTs were subjected to different treatments. For the treatment with glutaraldehyde, 4 mg of oxidized MWCNTs was mixed with 0.75 ml of a 5% glutaraldehyde solution dissolved in a 50 mM phosphate buffer (pH 7.0). This mixture was stirred for 2h. Then, the modified MWCNTs (MWCNTox_Y+GLU) were centrifuged and washed several times. Another treatment used was the functionalization with APTS. In this case, 15 ml of a 1% aqueous solution of APTS was added to 0.1 g of oxidized MWCNTs under stirring for 24 hours at 50 °C. After this, the nanotubes were filtered on a glass frit, washed with water until the filtrate reached neutral pH, and dried overnight at 105 ºC (MWCNTox_Y+APTS).

A third treatment was performed by combining the two treatments described above, i.e. first the functionalization with APTS and then with glutaraldehyde as described before (MWCNTox_Y+APTS+GLU).

Finally, it was carried out the functionalization with 0.75 ml of NHS solution with a concentration of 40 mg/l and 0.75 ml of EDC solution with a concentration of 20 mg/ml in 4 mg of oxidized MWCNTs under stirring for 1h. After that, the carbon nanotubes were centrifuged and washed several times (MWCNTox_Y+EDC/NHS).

3.3. Laccase immobilization technique

To immobilize laccase, an enzyme solution with the concentration of 3.75 µl/ml was prepared in phosphate buffer at pH 8. Thereafter 1.2 mL of this solution was introduced in an eppendorf along with 4 mg of carrier and stirred on an orbital shaker for 120 minutes. For the support treated with APTS and glutaraldehyde the immobilization time and temperature were 24h and 4 ºC, respectively. After immobilization, the MWCNT-laccase conjugate was recovered by centrifugation. Then, the material was successively washed with buffer, with the appropriate pH.

3.4. Enzymatic activity measurement

For the measurement of free laccase activity, 100 µL of enzyme solution was mixed with 0.5 ml 0.2 mM ABTS, and 1.4 mL of citrate/phosphate buffer 0.05/0.1M, pH 4.5 at 40 °C. Then the absorbance/min was recorded in a UV-Vis spectrophotometer (JASCO V-560) at 420 nm. The free laccase activity was calculated using Equation 1:

$$\frac{U}{L} = \frac{abs/min \times f_{dilution} \times 10^6}{\varepsilon} \qquad \text{Eq. 1}$$

Where, abs/min is the absorbance per minute determined by linear regression, $f_{dilution}$ is the dilution factor of the sample, 10^6 is the conversion factor from M to µM, ε is the molar extinction coefficient of ABTS (36000 $M^{-1}cm^{-1}$ at 420 nm) and U/l is the quantity of enzyme capable of oxidized down 1 µmol of ABTS per minute and per volume unit of enzymatic solution.

To measure the activity of the immobilized enzyme a solution with 37.5 ml of 0.4 mM ABTS, 105 mL of citrate/phosphate buffer 0.05/0.1M, pH 4.5 to 40°C was used. Subsequently, the immobilized enzyme was added to this solution under stirring. Samples were taken every 30 seconds for 2 minutes to measure the absorbance at 420 nm. Having made the linear regression, the enzymatic activity was determined by the following equation:

$$\frac{U}{g} = \frac{abs/min \times f_{dilution} \times V_{reaction} \times 10^6}{\varepsilon \times m_{carrier}} \qquad \text{Eq. 2}$$

Where, U/g is the quantity of enzyme capable of breaking down 1 μmol of ABTS per minute and per mass unit of carrier, abs/min is the absorbance per minute determined by linear regression, $f_{dilution}$ is the dilution factor of the sample, $V_{reaction}$ (L) is the volume of reaction, 10^6 is the conversion factor from M to μM, ε is the molar extinction coefficient (36000 $M^{-1}cm^{-1}$ at 420 nm) and $m_{carrier}$ (g) is the mass of MWCNTs with enzyme immobilized.

3.5. Determination of immobilization yield and recovered activity

The immobilization of laccase on the modified MWCNT samples, prepared by the different techniques described in section 3.2, was carried out in citrate/phosphate buffer 0.05 M/0.1 M (pH 4.5). Once the immobilization is finished, the MWCNT-laccase conjugates were tested towards ABTS oxidation.

In all the experiments, measurements of enzymatic activity were performed for the laccase solution used, the supernatants recovered after immobilization and the immobilized enzyme, according to the method mentioned above. The immobilization efficiency and the recovered activity after immobilization were also calculated for all the cases, according to Eq. 3 and Eq. 4, respectively.

$$\text{Immobilization efficiency} = \frac{activity_{free} - activity_{supernatant}}{activity_{free}} \times 100 \qquad \text{Eq. 3}$$

$$\text{Recovered activity} = \frac{\text{activity}_{\text{immobilized}} \times \text{m}_{\text{carrier}}}{(\text{activity}_{\text{free}} - \text{activity}_{\text{supernatant}}) \times \text{V}_{\text{solution}}} \times 100 \qquad \text{Eq. 4}$$

where $\text{activity}_{\text{free}}$ (U/l) is the enzymatic activity registered with a sample of the solution of laccase prepared to perform immobilization, $\text{activity}_{\text{supernatant}}$ (U/l) is the enzymatic activity registered with a sample of the supernatant recovered after performing immobilization, $\text{activity}_{\text{immobilized}}$ (U/g) is the enzymatic activity registered with the MWCNTs onto which laccase was immobilized, $\text{m}_{\text{carrier}}$ (g) is the mass of MWCNTs with enzyme immobilized and $\text{V}_{\text{solution}}$ (L) is the volume of enzyme used.

In this work, the immobilization efficiency represents the yield of the immobilization process in terms of the quantity of laccase adsorbed onto carbon nanotubes, while the recovered activity represents the yield of the immobilization process in terms of the capacity of the enzymes immobilized onto the carrier to perform catalysis.

3.6. Thermal stability of free and immobilized laccase

The thermal stability of the free and immobilized laccase was investigated by incubating the free and immobilized enzyme in phosphate (100 mM) buffer pH 8.0 at different temperatures (40-60 ºC). Immobilized laccase was suspended in 3 mL of the buffer. The sample was removed regularly from the water bath and enzymatic activity was quickly determined according to the methods described above.

The thermal parameters were calculated according to a simplified deactivation model described in the literature (Henley and Sadana 1985, Arroyo, Sánchez-Montero et al. 1999):

$$E \overset{k_1}{\rightarrow} E_1 \overset{k_2}{\rightarrow} E_2 \qquad \text{Eq. 6}$$

$$A = \left[100 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right] e^{-k_1 t} + \left[\frac{\alpha_2 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right] e^{-k_2 t} + \alpha_2 \qquad \text{Eq. 7}$$

where A (%) is the residual enzyme activity, α_1 and α_2 ratios of specific activities (remaining activities), respectively to the different states E_1/E and E_2/E (see Eq. 6), k_1(h^{-1}) and k_2 (h^{-1}) the thermal inactivation rate constants and t (h) *the* time.

Analyzing the data obtained, it was considered that laccase undergoes a conformational transition due to the increase in temperature according to the model of Eq. 6. This implies that inactivation follows a single exponential decay, in which $\alpha_2 = 0$ and $k_2 = 0$, leading to:

$$A = [100 - \alpha] \times e^{-kt} + \alpha \qquad \text{Eq. 8}$$

The thermal parameters α and k of the model described in Eq. 8 were estimated by a non-linear fitting of the experimental data, using CurveExpert v 2.0.4 © 2011-2014.

Biocatalyst half-life $(t_{1/2})$ was calculated from Eq. 8, using the estimated parameters (k and α) and making A equal to 50.

3.7. Characterization of MWCNTs

The textural characterization of the materials was based on the corresponding N_2 adsorption–desorption isotherms at 77 K, measured on a Quantachrome NOVA 4200e apparatus. Samples were first degassed in vacuum for 3 h at 393 K before analysis. The Brunauer–Emmett–Teller (BET) specific surface area (S_{BET}) was determined from the nitrogen adsorption data within the 0.05–0.15 range of relative pressure. Also the Fourier transform infrared (FTIR) analysis was performed on a FT-IR Nicolet 510-P spectrometer (Thermo Fisher Scientific, USA) equipped with a MIRacle™ Single Reflection ATR (Attenuated Total Reflectance ZnSe crystal plate) accessory (PIKE Technologies, USA).

3.8. Reutilization of the immobilized laccase

In order to investigate the reusability of the immobilized enzyme, the MWCNT-laccase bioconjugate was tested for ABTS oxidation and the enzymatic activity determined. Then, the material was recovered by filtration and thoroughly washed with buffer solution. Then, it was added to a fresh substrate solution, and the biocatalytic activity

determined. The activity of the immobilized enzyme after the first cycle was defined as the control and attributed a relative activity of 100%. Each cycle is defined here as the complete oxidation of the substrate (ABTS) present in a reaction mixture. This process was carried out for 5 cycles.

3.9. Phenol degradation

Phenol degradation was evaluated using free laccase and laccase immobilized on MWCNTs functionalized with EDC and NHS. ABTS was used as reaction mediator. A reaction solution (20 mL) containing a phenol concentration of 10 mg/l, a laccase concentration of 1 μL/ml and a ABTS concentration of 0.1 mM, was incubated for free laccase tests. For the tests with immobilized laccase, it was used the same concentration of phenol and ABTS and the immobilization process was similar to the process described above. The concentration of immobilized laccase was 1 mg/mL of solution. Several samples were taken in order to understand the effect of the mediated system (laccase+ABTS) on phenol degradation. The samples (0.7 mL) were added to a solution (70 μL) of H_2SO_4 2 M for stopping the reaction. Then the concentration of phenol was analyzed by high performance liquid chromatography (HPLC) using a Hitachi Elite LaChrom apparatus equipped with a L-2450 diode array detector. The stationary phase consisted in a Purospher Star RP-18 endcapped column (250 mm × 4.6 mm, 5 μm particles) working at room temperature. The method starts with an equilibrated mixture of water (A):methanol (B) (70:30) at a flow rate of 1 mL min^{-1} followed by a linear gradient step to A:B (47:53) in 16 min; finally the initial conditions were re-established in a 1 min gradient step and the A:B (70:30) mixture was isocratically eluted for 6 min. Two different concentrations of free laccase were tested. Immobilized laccase reutilization was assessed.

4. RESULTS AND DISCUSSION

4.1. Effect of MWCNTs functionalization on laccase immobilization and catalytic activity

4.1.1. MWCNTs functionalized with carboxylic acid groups

Carbon nanotubes, as shown in various studies (Matsuura, Saito et al. 2006, Nepal and Geckeler 2006, Gao and Kyratzis 2008), are capable of adsorbing various kinds of compounds, including enzymes. This adsorption occurs through electrostatic and hydrophobic interactions (Karajanagi, Vertegel et al. 2004) thus giving rise to a non-covalent bond. According to some authors, other interactions are also important for enzyme adsorption in MWCNTs. Interactions such as hydrogen bonds and nonspecific adsorption (π-π interactions) are something to consider (Matsuura, Saito et al. 2006). However, it is necessary to take into account the great influence of the surface of carbon nanotubes has in enzyme immobilization. MWCNTs were oxidized with HNO_3 with different concentration. This treatment leads to the formation of mostly carboxylic acid groups, but phenol, quinone and carbonyl groups are also formed in a less extent at the surface of carbon nanotubes (Figueiredo, Pereira et al. 1999, Figueiredo, Pereira et al. 2006, Figueiredo and Pereira 2010, Silva, Tavares et al. 2014).

As shown in Table 7, it was obtained a high value of enzyme immobilization yield (96%) for carbon nanotubes treated with a higher acid concentration (0.3 M).

Table 7 - Laccase immobilization yield and recovered activity for MWCNTs treated with different acid concentrations.

Support	Immobilization yield (%)	Recovered activity (%)
MWCNTox_0.05M	83.4	14.6
MWCNTox_0.1M	84.5	13.1
MWCNTox_0.2M	90.8	16.5
MWCNTox_0.3M	96.0	15.7

The immobilization yield appeared to be directly proportional to the HNO_3 concentration used for the oxidizing treatment. This can be explained by the fact that the use of nitric acid in the purification process causes both, purity improvement as well as enhanced oxidation of MWCNT surface leading to a better enzyme immobilization.
In respect to the recovered activity, the values obtained for the samples prepared with different acid concentrations were all very similar varying in the range of 13-17% (Table 7). The recovered activity (16.5%) was obtained for the support treated with HNO_3 0.2 M. Compared with other treatments, in this case, it may be said that the recovered activity is not directly proportional to the immobilization yield. This suggests that, although we have more immobilized enzyme, it does not always active in carbon nanotubes. One explanation for this, was that the immobilization of the enzyme on the surface of carbon nanotubes leads to a change in its conformation and thus can lead to inactivation of the enzyme.

4.1.2. MWCNTs functionalized with carboxylic acid groups and APTS

MWCNTox were treated with 3-aminopropyltriethoxysilane (APTS) as described in section 3.2. This compound is a silane that contains an amino-group. Once the amine is available, numerous crosslinking agents can be used to immobilize proteins, DNA or other molecules on the surface of many supports. This treatment will not provide a covalent immobilization, however the results (Table 8) showed an increase on the immobilization yield, comparing with the results described in section 4.1.1.

Table 8 - Laccase immobilization yield and recovered activity for MWCNTs treated with different acid concentrations and APTS.

Support	Immobilization yield (%)	Recovered activity (%)
MWCNTox_0.05M+APTS	98.5	6.0
MWCNTox_0.1M+APTS	99.2	6.8
MWCNTox_0.2M+APTS	99.5	7.0
MWCNTox_0.3M+APTS	99.8	16.1

The treatment with APTS may result in stabilization of the enzyme on the support leading probably to an increase of electrostatic and hydrophobic interactions. The percentage of yield immobilization was around 99% for all supports. The highest value of the recovered activity was obtained for the carbon nanotubes oxidized with the highest concentration of acid (0.3M). Nevertheless, comparing with the previous treatment the recovered activity decreased for the materials prepares with lower HNO_3 concentration, getting values around 6-7% (Table 8).

4.1.3. MWCNTs functionalized with carboxylic acid groups and glutaraldehyde

Glutaraldehyde activation of supports is one of the most popular techniques to immobilize enzymes (Silva, Silva et al. 2007). The methodology is quite simple and efficient and, in some instances, it even allows to improve enzyme stability by multipoint or multisubunit immobilization (Vaillant, Millan et al. 2000, Isgrove, Williams et al. 2001).

In this section the oxidized MWCNTs were treated with glutaraldehyde in order to obtain a covalent laccase immobilization. In this case the proteins are mostly immobilized through their amine groups. There are two main types of amine groups exposed to the medium: ε-amine groups of lysine residues and terminal amine-groups. The use of glutaraldehyde for covalent immobilization can be carried out in several ways. Immobilization of enzymes on supports previously activated with glutaraldehyde or pre-adsorption of proteins onto supports with primary amino groups followed by the treatment with glutaraldehyde are two possible alternatives (Alonso, López-Gallego et al. 2005).

In the present work, MWCNTox_Y were activated with glutaraldehyde. From Table 9, the immobilization yield decreased compared to the oxidation treatment, for example, MWCNTox_0.3M and MWCNTox_0.3M+GLU obtained 96% and 85.7% of immobilization yield, respectively. One of the reasons for this result was probably the occupation of the carbon nanotubes surface with the molecule of glutaraldehyde leading to a decrease in the surface available for immobilization by adsorption and therefore a decrease of the immobilization yield. Nevertheless, the materials treated with glutaraldehyde are expected to establish a stronger interaction with the enzyme.

Table 9 - Laccase immobilization yield and recovered activity for MWCNTox_Y treated with glutaraldehyde.

Support	Immobilization yield (%)	Recovered activity (%)
MWCNTox_0.05M+GLU	62.5	9.9
MWCNTox_0.1M+GLU	67.6	9.1
MWCNTox_0.2M+GLU	71.1	10.0
MWCNTox_0.3M+GLU	85.7	16.4

In this case, as in the previous results, there was a higher immobilization yield and recovered activity for the carbon nanotubes treated with higher acid concentration. This behavior should be related to a higher availability of carboxylic acid groups may interact with glutaraldehyde.

4.1.4. MWCNTs functionalized with carboxylic acid groups and APTS and glutaraldehyde

Glutaraldehyde has also been used to introduce intermolecular crosslinking in proteins (Silva, Silva et al. 2007) or to modify adsorbed proteins on aminated supports (Rodríguez Couto, Sanromán et al. 2004). The exact structure of glutaraldehyde on the support is still under discussion (Migneault, Dartiguenave et al. 2004, Betancor, López-Gallego et al. 2006), but given the high stability of the amino-glutaraldehyde bond, the formation of some kind of cycle seems to be a likely possibility. For this happens, the support was previously treated with 3-aminopropyltriethoxysilane (APTS) in order to obtain free amino-groups. Then the glutaraldehyde will activate the aminated support for the enzyme covalently binds. Unlike the expected, results were not the best (Table 10). Either immobilization yield or the recovered activity decreased compared with the previously results.

Table 10 - Laccase immobilization yield and recovered activity for MWCNTox_Y treated with APTS and glutaraldehyde.

Support	Immobilization yield (%)	Recovered activity (%)
MWCNTox_0.05M+APTS/GLU	66.8	2.9
MWCNTox_0.1M+ APTS/GLU	64.7	2.3
MWCNTox_0.2M+ APTS/GLU	71.6	2.8
MWCNTox_0.3M+ APTS/GLU	72.4	8.3

One explanation for these results is that the enzyme immobilization was only driven by covalent bonding, not occurring immobilization by adsorption. Therefore there is less enzyme immobilized on the carbon nanotubes but it is expected that the interaction between the enzyme and the support to be stronger. As expected, the best results were obtained for the material prepared with the carbon nanotubes oxidized with the highest HNO_3 concentration, with 71.4% and 8.3% for immobilization yield and recovered activity, respectively. However, as the recovered activity was so low, this treatment was discarded to further tests as would be difficult to apply this immobilization technique to biocatalytic processes.

4.1.5. MWCNTs functionalized with carboxylic acid groups and EDC/NHS

The final approach was the treatment with EDC and NHS. EDC is a zero-length cross-linker widely used in protein conjugations. The conjugation reactions occur in two sequential steps (Fig. 17). EDC first reacts with a carboxylic acid group, forming an amine-reactive *O*-acylisourea intermediate which subsequently reacts with an amine group to produce a stable amide bond. However, the *O*-acylisourea intermediate is very unstable and susceptible to hydrolysis. Such instability results in low coupling efficiency. The addition of NHS (or its more water soluble analogue Sulfo NHS) stabilizes the intermediate by converting it to a semistable amine-reactive NHS ester, thus increasing the coupling efficiency by 10-20 fold (Staros, Wright et al. 1986, Sehgal and Vijay 1994).

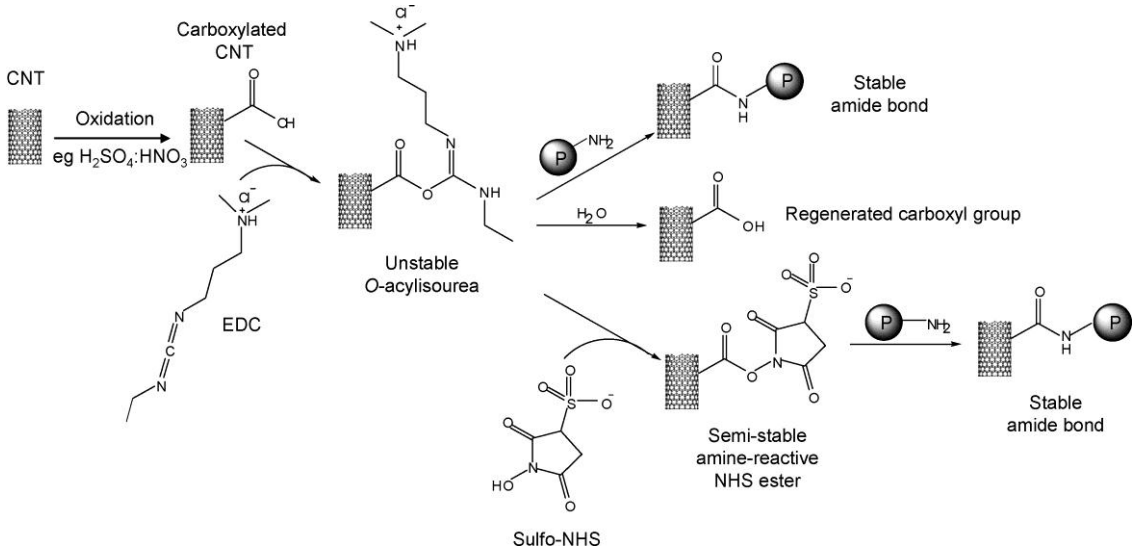

Fig. 17 - Theoretical conjugation of proteins to carboxylated CNTs using EDC in the presence or absence of sulfo-NHS.

The results presented in Table 11 show that this technique resulted in 100% immobilization yield for all the supports used. Among all the materials tested for laccase immobilization, the highest value of recovered activity (of 20.5%) was obtained for the MWCNTox_0.3M+EDC/NHS.

Table 11 - Laccase immobilization yield and recovered activity for MWCNTox_Y treated with EDC/NHS.

Support	Immobilization yield (%)	Recovered activity (%)
MWCNTox_0.05M+EDC/NHS	99.7	8.4
MWCNTox_0.1M+ EDC/NHS	99.7	9.4
MWCNTox_0.2M+ EDC/NHS	99.8	9.8
MWCNTox_0.3M+ EDC/NHS	99.8	20.4

These results indicate that the enzyme is probably covalently bonded to the support, improving the laccase immobilization capacity and consequently increase the immobilization yield and the recovered activity.

Comparing all the different modified MWCNT supports, it can be concluded that, in terms of immobilization yield, the best results were obtained by treating MWCNTox with APTS and EDC/NHS (Fig. 18).

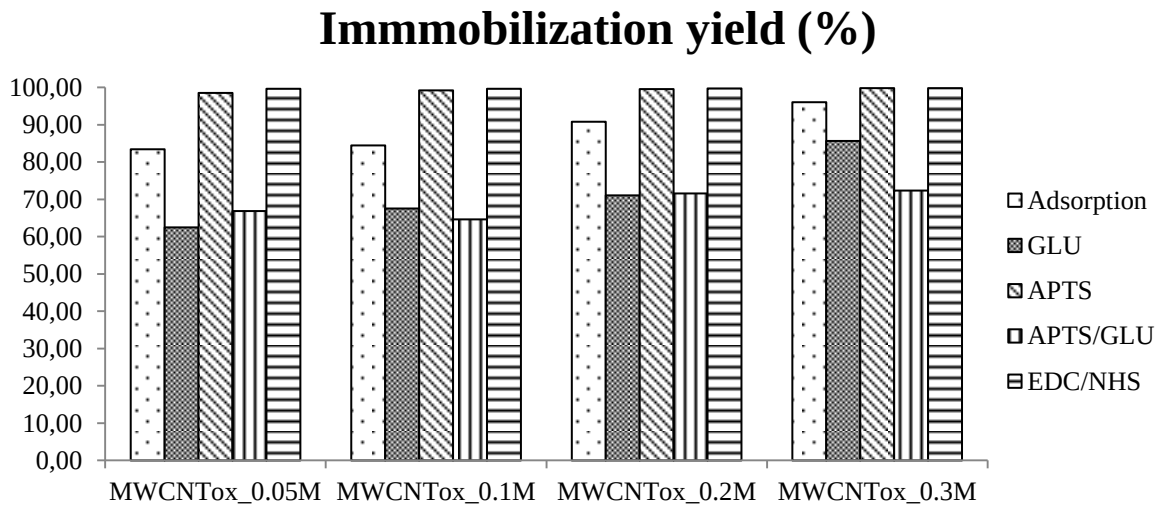

Fig. 18 - Immobilization yield for MWCNTs treated with different acid concentrations and the different functionalization.

Regarding the recovered activity, the best results were in general obtained for the oxidized MWCNT supports (MWCNTox) in which the enzyme is immobilized by direct physical adsorption (Fig. 19). In this case, the enzyme may be more active, however the enzyme-support bond is weaker, which may be a drawback in what concerns reutilization. From the covalent approaches, the best results were obtained with the EDC/NHS treatment, since a stronger interaction between the enzyme and the support is expected, which foresee that this material is a good candidate for being used in successive runs without losing its performance Finally, it can be concluded that the best oxidation process was that using HNO_3 concentration probably due to the higher abundance of carboxylic acids at the surface of the MWCNTs. For this reason, the further tests were performed only with MWCNTs oxidized with HNO_3 0.3 M.

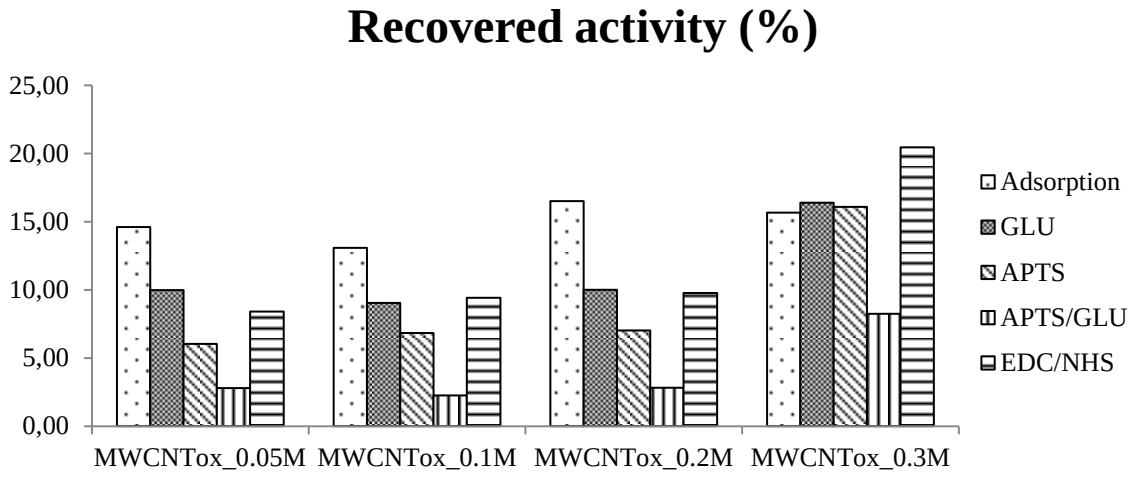

Fig. 19 – Recovered activity for MWCNTs treated with different acid concentrations and the different functionalization.

4.2. Thermal stability of free and immobilized laccase

The temperature was one of the factors that were analyzed to determine the stability of the enzyme immobilized on the modified carbon nanotubes. Enzymes, such as laccase, suffer from the effects of temperature on its conformation and also on the enzyme-substrate complex that affects its catalytic performance. It is important to know if the immobilization of the enzyme on the carbon nanotubes modified different approaches will lead to enhanced stability. The tests were performed at three different temperatures (40 ºC, 50 ºC and 60 ºC). From Fig. 20, it can be observed that the data follows an exponential decay model (Eq. 8). In this model the enzyme was inactivated in only one phase (the one-step transition between the active and denatured state) with the possibility of the existence of remaining activity at the end, which is represented by the parameter α.

As expected, the increase in temperature leads to a decrease in the stability of the enzyme and the reduction of its activity. This inactivation is probably due to the higher vibration of the laccase structure that may break some chemical bonds inside the molecule and as consequence, change its 3D structure (Silva, Tavares et al. 2014). For the material modified with EDC/NHS, it can be observed in Fig. 20 that after a first decay in activity when incubated at 40 °C, the performance was nearly constant at c.a. 93%. As the temperature increases, the activity decreases, being of 84% and 71% for the tests performed at 50 °C and 60 °C , respectively, after 4 hours of incubation. A good thermal stability was also obtained for the material functionalized with APTS. The values for the thermal stability at 40 °C are quite similar to the values of the treatment with EDC/NHS. For the other temperatures tested, there was a decreasing of thermal stability. Once again and as already mentioned, the functionalization with APTS may have increased the interaction of the enzyme probably through hydrophobic and electrostatic interactions with the surface of the modified carbon nanotubes. On the other hand, the APTS molecules may have functioned as a protection for the enzyme.

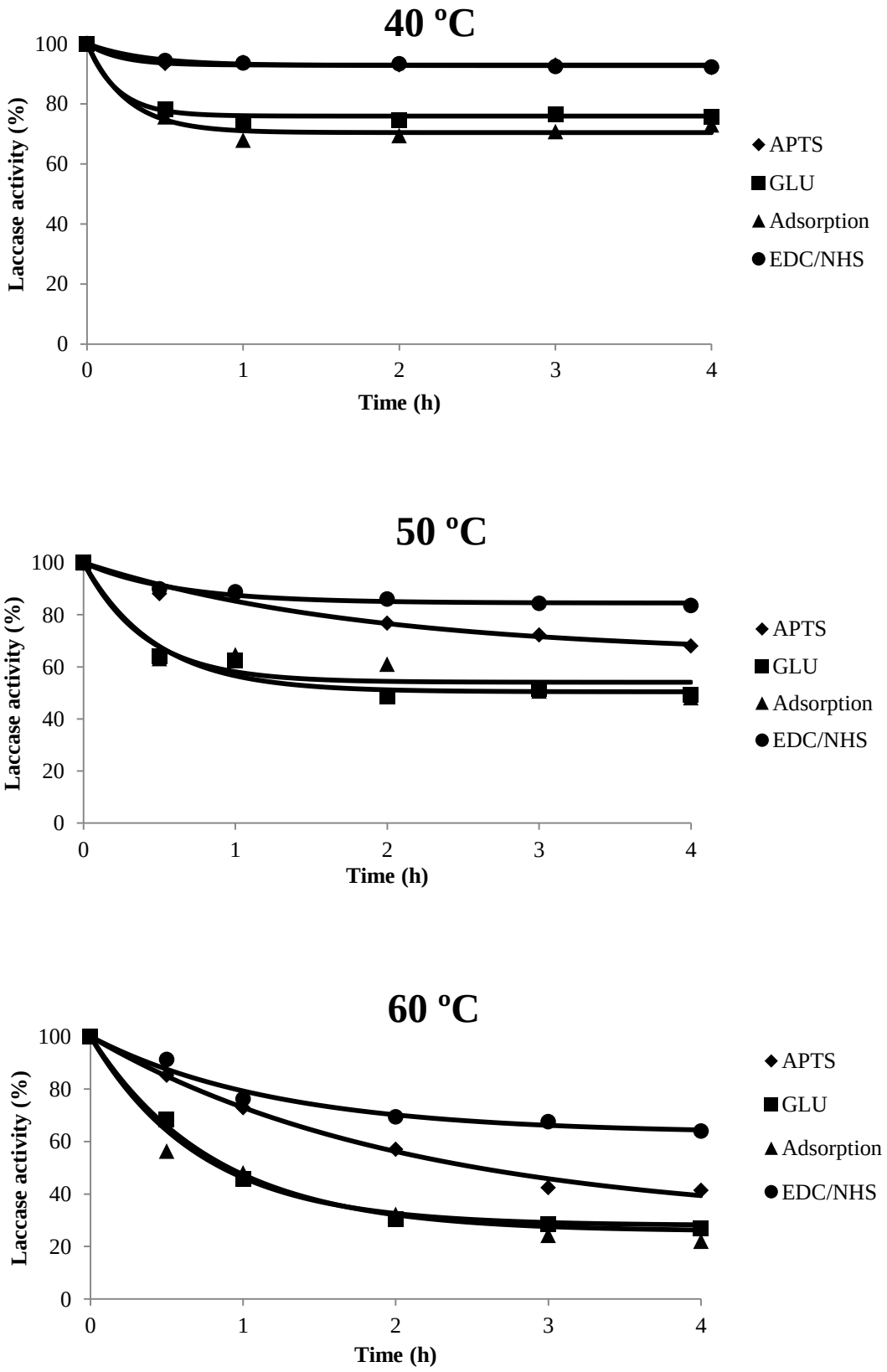

Fig. 20 - Thermoinactivation of immobilized laccase at temperatures from 40 to 60ºC.

The kinetic thermal parameters were also obtained from experimental data in order to evaluate the thermal stability of the enzyme. The parameters referring to the different approaches of functionalization are presented in Table 12. As observed, for the temperature of 60 ºC, the parameter α (remaining enzyme activity) is almost 2 times higher for treatment with EDC/NHS regarding to the other treatments, confirming the enhanced performance of the material produced by this route. Regarding the parameter *k*, it is higher for the oxidized support (MWCNTox) and that treated with GLU, with *k* values of 1.38 and 1.23 respectively. Thus indicating that there was a more rapid initial inactivation for the bioconjugates obtained from these treatments. Comparing the treatment of APTS and EDC/NHS, it can be seen that *k* is higher for EDC/NHS. However, the value of α was 2 times higher, with an increase from 28.9 to 63.0 for functionalization with APTS and EDC/NHS, respectively. This means that, as expected, the binding of the enzyme to the functionalized carbon nanotubes with EDC/NHS is stronger and more stable. Regarding the $t_{1/2}$, the enzyme immobilized on MWCNTox_0.3M and MWCNTox_0.3M+GLU lost 50% of its initial activity after 0.86 h and 0.91 h of incubation at 60 °C, respectively, whereas for the immobilized laccase on MWCNTox_0.3M+APTS, $t_{1/2}$ was 2.54 h. On the other hand, the activity of laccase immobilized on MWCNTox_0.3M+EDC/NHS remained above 50% from its initial value and therefore no half life time could be determined due to their high thermal stability.

Table 12 - Thermal parameters for immobilized laccase.

Thermal parameters									
Support	**40 ºC**			**50 ºC**			**60 ºC**		
	α	**k (h^{-1})**	**$t_{1/2}$ (h)**	**α**	**k (h^{-1})**	**$t_{1/2}$ (h)**	**α**	**k (h^{-1})**	**$t_{1/2}$ (h)**
MWCNTox_0.3M	70.40	3.89		54.08	2.44		27.98	1.38	0.86
MWCNTox_0.3M+GLU	70.89	5.20	-	50.42	2.10	-	25.75	1.23	0.91
MWCNTox_0.3M+APTS	92.88	4.73	-	64.59	0.54	-	28.94	0.48	2.54
MWCNTox_0.3M+EDC/NHS	92.71	2.71	-	84.52	1.64	-	63.04	0.82	-

Hereupon, looking at the effects of different approaches, it was concluded that the enzyme immobilized on MWCNTox functionalized with EDC/NHS presents the highest thermal stability. The increase of thermal stability observed may be attributed to the reduction in the protein structure mobility, due to anchorage to the support and subsequent translation of the rigidity at each anchorage point to the whole enzyme structure, thus protecting it from the denaturing effects of the environment (Verma, Naebe et al. 2013).

Thermal stability was compared for free enzyme and immobilized enzyme. From Fig. 21, it can be stated the thermal stability increased for the immobilized enzyme. For example, at 60ºC, free laccase loses about 57% of activity after 4 hours while the immobilized enzyme only loses 38%.

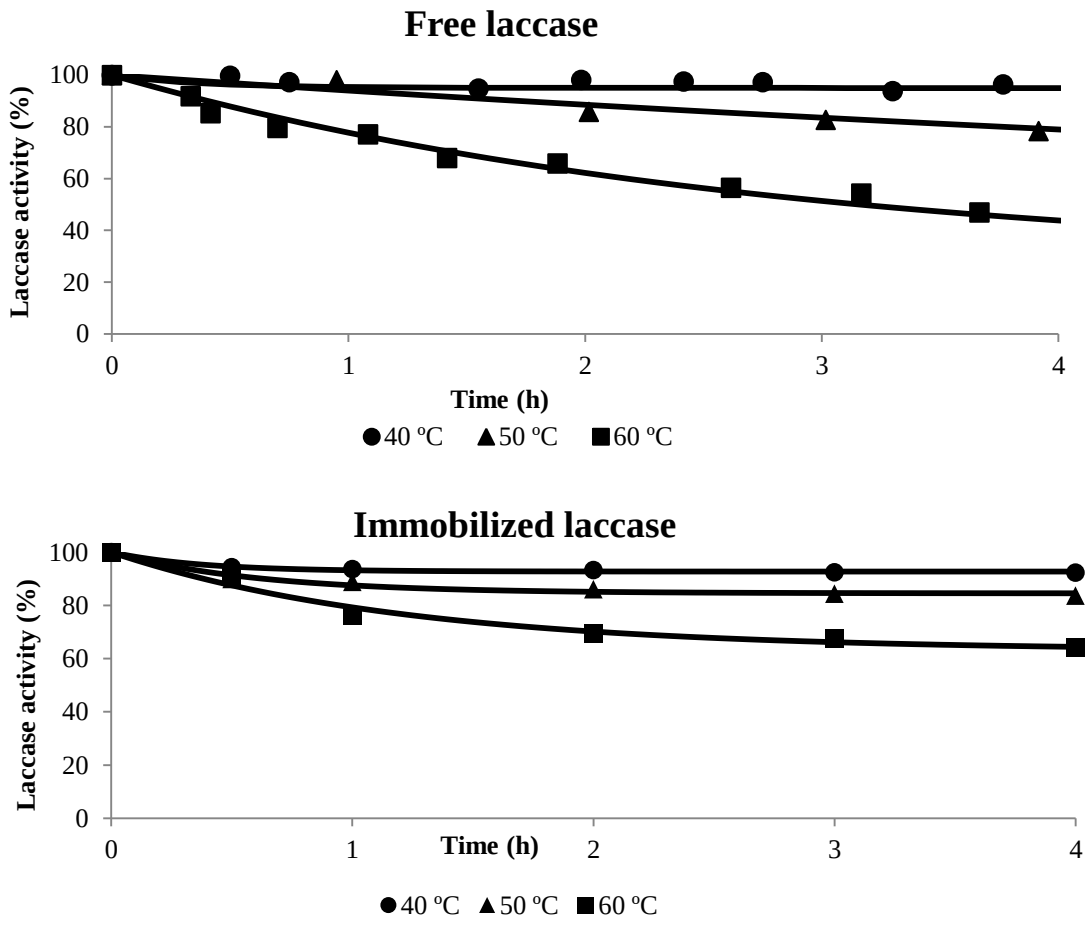

Fig. 21 - Thermoinactivation of free laccase and immobilized laccase at temperatures from 40 to 60 ºC.

The thermal parameters confirm the results described above (Table 13). Comparing free and immobilized laccase, the α parameter being higher for immobilized enzyme at 50 °C and 60 °C. The value of α was about 2.64 and 2.28 times the value of free enzyme and for the temperatures of 50 ºC and 60 ºC respectively. Regarding the *k* value, the values were higher for the immobilized enzyme, indicating, once again, that there was a fastest initial inactivation. In conclusion, as expected, the laccase immobilized on MWCNTox_0.3M_EDC/NHS was much more stable, indicating that the enzyme-support complex probably causes a decrease in the mobility of the protein structure due to multi-point attachment of the enzyme to the support.

Table 13 - Thermal parameters for free and immobilized laccase at temperature from 40 to 60 ºC

	Free laccase			**Immobilized laccase - MWCNTox_0.3M_EDC/NHS**		
T (ºC)	**k (h^{-1})**	**α**	**$t_{1/2}$ (h)**	**k (h^{-1})**	**α**	**$t_{1/2}$ (h)**
40	-	-	-	2.71	92.71	-
50	0.09	32.00	14.40	1.64	84.52	-
60	0.36	26.50	3.16	0.82	63.04	-

4.3. Reutilization tests

One of the great potential of enzyme immobilization is the ability to being reused. The main advantage for enzyme immobilization, contrary to what happens with free enzyme, is the possibility of the enzyme to be recovered. This can be achieved by simple processes as filtration or centrifugation. After separating the enzyme from the reaction mixture (substrates and products) it can be reused several times, which reduces the enzyme and the enzymatic products cost tremendously.

As already mentioned, various methods of enzyme immobilization were tested. Non-covalent immobilization methods, which are based mostly on hydrogen bonding and electrostatic interactions, lead to a weaker type of immobilization. Covalent immobilization, which is based on the interaction between the enzyme and cross-linkers, leads to a stronger immobilization that, potentially leads to a higher enzyme activity during several cycles of reuse. In this section the results of immobilized laccase reutilization for the different functionalization approaches are presented. Fig. 22 reveals that after 5 cycles of utilization, the highest activity was obtained for carbon nanotubes functionalized with EDC/NHS. The remained value of the enzyme activity for this material after 5 cycles was 66% in contrast to 38% for that obtained from the treatment with APTS and 29% and 18% for MWCNTox_0.3M and MWCNTox_0.3M+GLU, respectively. As previously discussed, MWCNTox functionalization with EDC/NHS promotes the covalent immobilization between laccase and support. However, it should also be noticed a loss of 44% of the enzyme activity. This activity decrease can be explained by the loss of material throughout the several cycles since the tests are being carried out with a low amount of support (5 mg). For the other materials the results were a little different than expected. At the end of the first reuse there was a loss of about 64% of the enzyme activity in the carbon nanotubes treated with glutaraldehyde. These results for MWCNTox_0.3M+GLU corroborate the results obtained in the last section for thermal stability. The results obtained for MWCNTox_0.3M show a steady decline over the five cycles. Nevertheless, at the end of the first and second reuse, the enzymatic activity values were higher in comparison with MWCNTox_0.3M+GLU and MWCNTox_0.3M+APTS. For MWCNTs_ox_0.3M+APTS, although there is a great activity loss immediately after the first recycling (57%), it is noticed that the enzyme activity remained in the range 38-44% over the five cycles of reaction.

From the above results it can be concluded that the modification with EDC/NHS produce the best results in terms of operational and thermal stabilities.

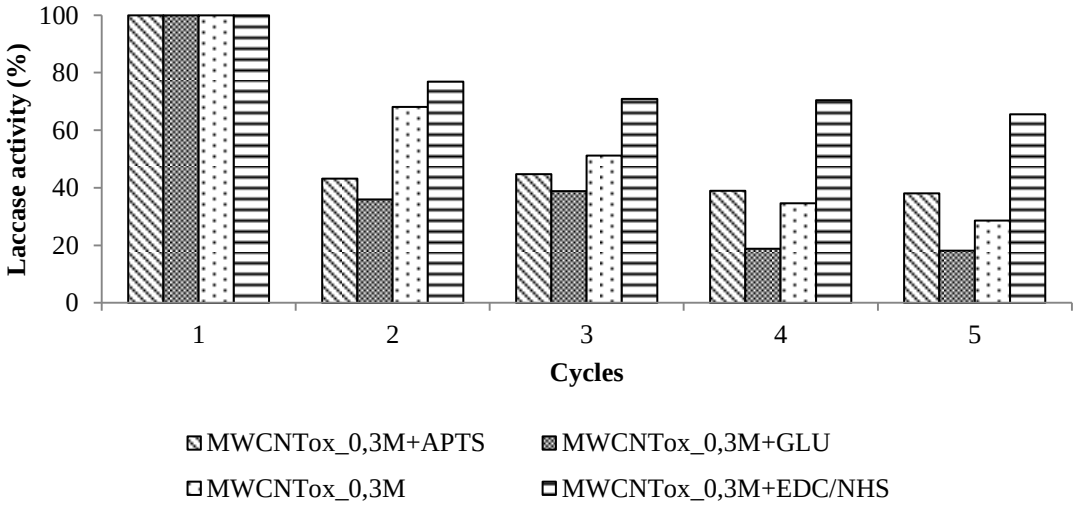

Fig. 22 - Laccase activity after 5 cycles of utilization.

4.4. Characterization of MWCNTs

In order to explain some of the previous results the characterization of the carbon nanotubes was performed. First, to understand the influence of the oxidation on carbon nanotubes, as mentioned above, the surface areas of the untreated MWCNTs and MWCNTox_0.3M were determined. The oxidation with HNO_3 produces hydrophilic carboxylic acid and hydroxyl groups along their sidewalls leading to a modification in the conformation of the nanotubes. The surface areas of the samples, calculated by the BET method (S_{BET}), revealed that the oxidation treatment led to an increase in the surface area of the resulting material. The surface area of the MWCNT sample treated with HNO_3 0.3 M (83 $m^2.g^{-1}$) is around 14% higher than that of pristine MWCNT (73 $m^2.g^{-1}$). This occurs because oxidative treatment with HNO_3 creates sidewall defects and can open up the end caps of the MWCNT, therefore increasing the porosity leading to a high immobilization yield.

The FTIR analysis of MWCNT, MWCNTox_0.3M and MWCNTox_0.3M+EDC/NHS with and without immobilized laccase was carried out in order to understand the interactions between the enzyme and the support.

Fig. 23 shows the FTIR-ATR spectra of pristine MWCNT and the sample oxidized with HNO_3 0.3 M, where five main bands can be identified (Chen, Chen et al. 2012). The band at 1800 cm^{-1} attributed to the C=O stretching vibration in lactones, carboxylic acids, and anhydrides. The peak at 1630 cm^{-1} is attributed to water adsorbed mainly on the MWCNTs (Chen, Chen et al. 2012). The broad band centered at 1400 cm^{-1} assigned to O−H bending in phenols and carbonyls and the band at 1020 cm^{-1} assigned to the C−O stretching vibration in ethers and phenols. Finally a band at 880 cm^{-1} ascribed to isolated aromatic C−H out-of-plane bending mode vibration is also observed.

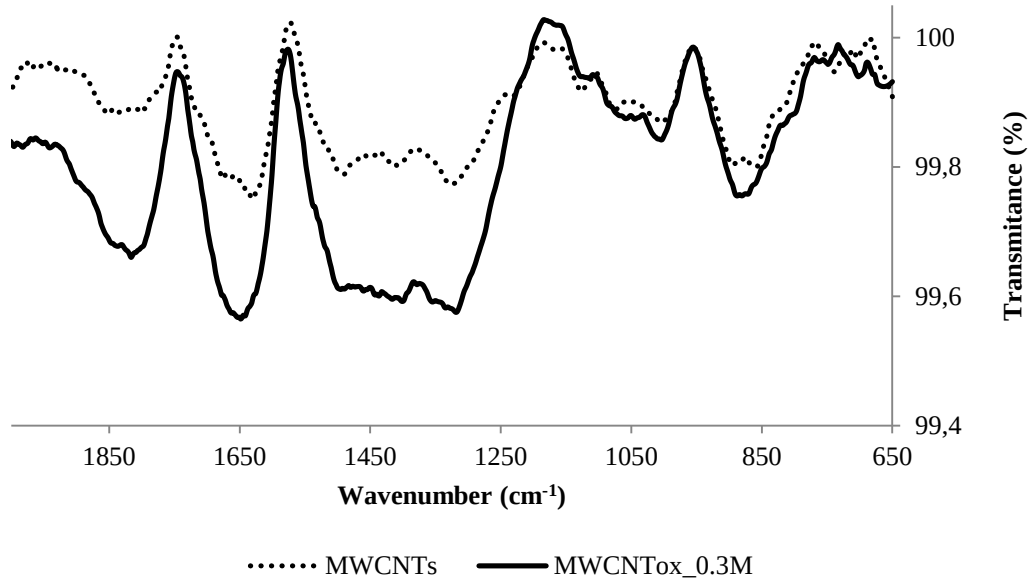

Fig. 23 - FTIR-ATR spectra of MWCNTs and MWCNTox_0.3M.

For every band the intensity increased to carbon nanotubes treated with acid. The intensity of the band at 1800 cm^{-1} and at 1400 cm^{-1} increased indicating the generation of carbonyls and carboxylic groups at the surface. This confirms the introduction of oxygenated functional groups at the surface of the MWCNT upon treatment with HNO_3. The presence of such groups, mainly carboxylic acids, increases the interaction between the enzyme and the carrier improving the immobilization of laccase.

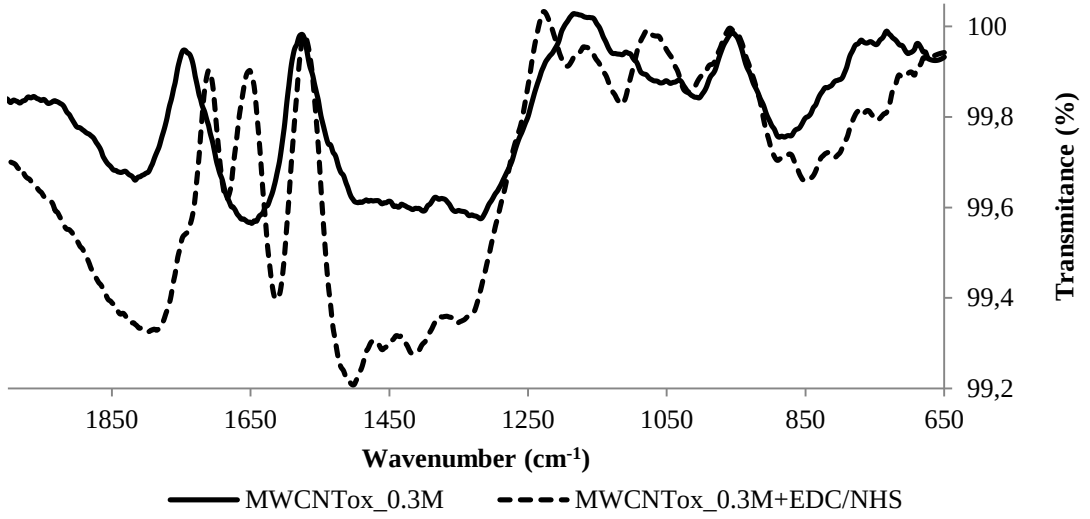

Fig. 24 - FTIR-ATR spectra of MWCNTox_0.3M and MWCNTox_0.3M+EDC/NHS.

After treatment with EDC/NHS it is possible to observe the appearance of two new bands at 1600 cm^{-1} and 1650 cm^{-1}. According to the literature (Sam, Touahir et al. 2009), these bands are related to peptide C=O stretch (amide I) and mostly peptide N−H bend (amide II) respectively. Another band that appeared at c.a. 1200 cm^{-1} which can also refer to a C−N vibration. It is noted an increase in the intensity of the band at 1800 cm^{-1} which indicates attachment of the succinimidyl ester termination to the surface. This intense band is actually the result of the overlap of different peaks, namely at 1820 cm^{-1}, which is related with suc-ester carbonyl stretch, at 1785 cm^{-1} attributed to the suc-cycle C=O symmetric stretch and at 1745 cm^{-1} corresponding to suc-cycle C=O antisymmetric stretch. These results confirm the functionalization of the oxidized carbon nanotubes with EDC/NHS treatment that is expected to promote the covalent attachment of the enzyme to the support.

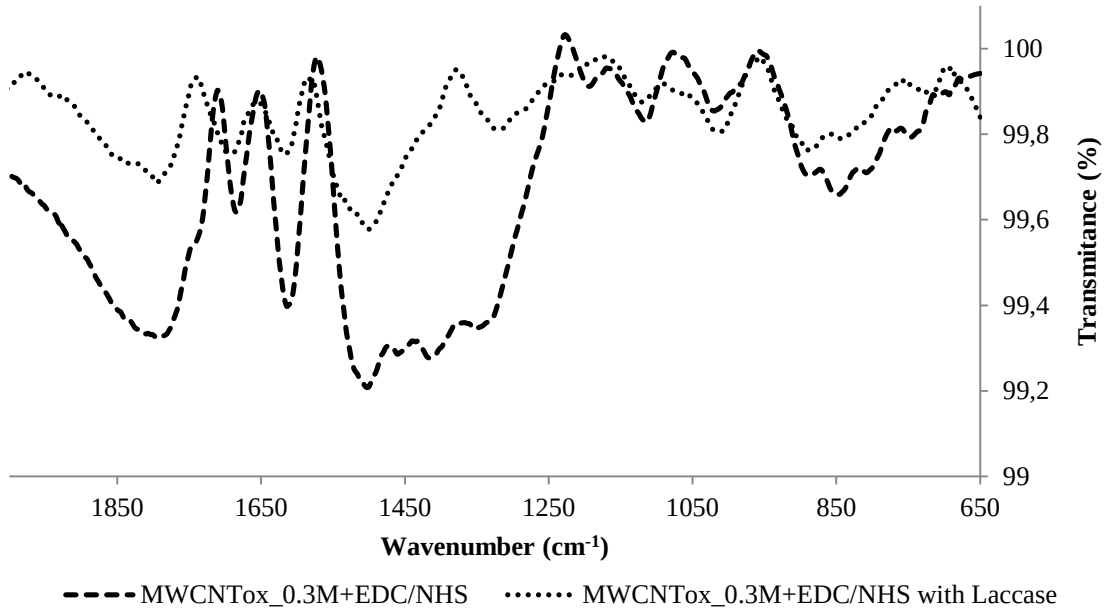

Fig. 25 - FTIR-ATR spectra of MWCNTox_0.3M+EDC/NHS before and after laccase immobilization.

Finally, looking at the spectrum of MWCNTox_0.3M+EDC/NHS with immobilized enzyme (Fig. 25) it can be seen that there was a decrease in the intensity of the bands at 1650 cm^{-1}, 1550 cm^{-1} and at 1200 cm^{-1}. This loss of intensity suggests that the enzyme bound to these functional groups. Furthermore it should be noticed the appearance of a new band at 1320 cm^{-1}, which according to literature refers to C−N stretching vibration of amines (Silva, Tavares et al. 2014). This may indicate that there was possibly of

some part of the enzyme adsorbed on the carbon nanotubes. With this, by the results obtained, it can be concluded that the enzyme bound to the functional groups that have been provided by functionalization with EDC/NHS.

4.5. Phenol degradation

The generation of a variety of molecules derived of industrial processes has negative impacts for ecosystems and humans. Due to this problem, research has been conducted to investigate the new possibilities of applying enzymes for organic waste treatment. It is known that several types of industrial and agricultural wastes contain phenolic compounds: paints, pesticides, wastes from coal conversion, polymeric resins and petroleum (Tavares, Pinho et al. 2012). Due to the high solubility of phenol, it is still difficult to achieve satisfactory removal efficiency by current treatment techniques (Saitoh, Sugiura et al. 2009). Enzymatic wastewater treatment is a promising technology for the degradation of phenol and phenolic compounds with high degree of specificity and minimal environmental impact (Gomez, Matafonova et al. 2009). The essential limitation of laccase used in degradation of phenol and phenolic compounds is its low stability and productivity, as well as its high production cost (Busca, Berardinelli et al. 2008). In order to reduce these limitations for enabling industrial application, laccase has been reported to be successfully immobilized on various carriers for improving biodegradation of phenol and phenolic compounds (Wang, Hu et al. 2012) .

In this work the biocatalytic degradation of phenol in aqueous solution was carried out using ATBS as mediator and laccase immobilized on MWCNTox_0.3M+EDC/NHS.

First, a test was made using free laccase and using bare MWCNTs to determine the ability of the laccase to degrade phenol without the any mediator and if phenol could adsorb to the carbon nanotubes. As can be seen from Fig. 26, after 60 minutes, phenol concentration remained practically unaltered in the presence of either free laccase or MWCNTs. These results indicate that a mediator is needed to activate laccase and also that there is no significant adsorption of phenol on MWCNTs.

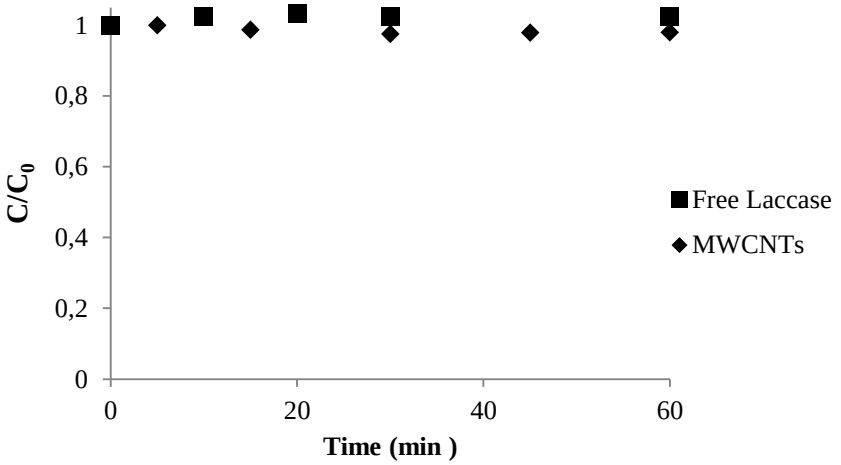

Fig. 26 - Phenol degradation and phenol adsorption using free laccase and MWCNTs (C_0 corresponds to the initial phenol concentration and C to the concentration at each reaction time).

Then, the mediated system (laccase+ABTS) was tested. Reactions were performed with 2 different enzyme concentrations, at a concentration of 1µl/ml (Laccase I) and at a concentration of 4µl/ml (Laccase II). Looking at the results obtained (Fig. 27) it can be concluded that the mediated system performed very well. After 60 minutes there was a phenol degradation of more than 90% for the two concentrations tested, indication that ABTS is a promising mediator. The necessity of a mediator is not surprising since the redox potential (E^0) of ABTS, i.e. 690 mV (Branchi, Galli et al. 2005), is lower than that of the T1 copper (785 mV) (Call and Mücke 1997). This yields a high ΔE^0 (Eq. 9) and, hence, a driving force for electron transfers between T1 copper and ABTS, which contributes to a high reaction rate. The rate of electron transfer from the substrate to the T1 copper in the active site of laccase is generally regarded as the rate-limiting step in the laccase-catalyzed reaction. The rate depends on the difference between the redox potentials (ΔE^0) of the T1 copper and the substrate (Xu 1996, Xu 1997) , as follows:

$$\Delta E^0 = E^0(T1\ copper) - E^0(\text{Substrate}) \qquad \text{Eq. 9}$$

On the other hand it should be also noticed that, as expected, an increase in the enzyme concentration, also leads to an increase in the reaction rate.

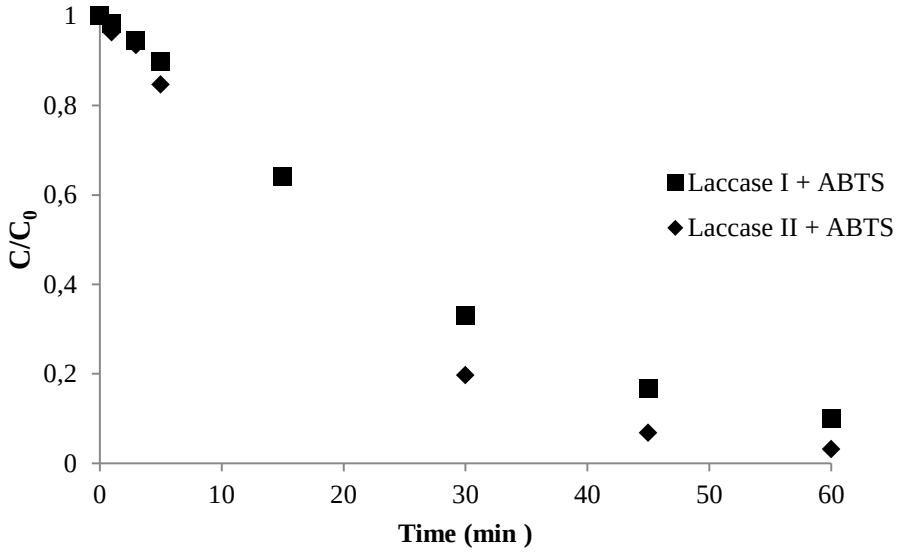

Fig. 27 - Phenol degradation using different concentrations of laccase.

Looking now at the results of the immobilized laccase (Fig. 28), it is possible to see that, as expected, the reaction was slower. As discussed previously, laccase loses some activity when immobilized on carbon nanotubes. After 1 hour of reaction it was obtained a degradation of about 47%. This value is about 2 times lower than the value obtained for the free enzyme. However, despite the decrease of the reaction rate, 87% of phenol degradation was obtained at the end of 4 hours. The immobilized enzyme was reused in a new catalytic run, starting with a fresh phenol solution. The results after reuse show a decrease in phenol degradation after 4 hours of reaction of 13%. This decrease in the catalytic activity may be attributed to some loss of material in the intermediate steps of centrifugation and filtration or also due to possible loss of enzyme to the solution. Nevertheless, the results for reuse can be considered very satisfactory with about 70% of phenol degradation being obtained after 4 hours of reaction. These are very promising results in what concerns the use of this system in real-scale applications.

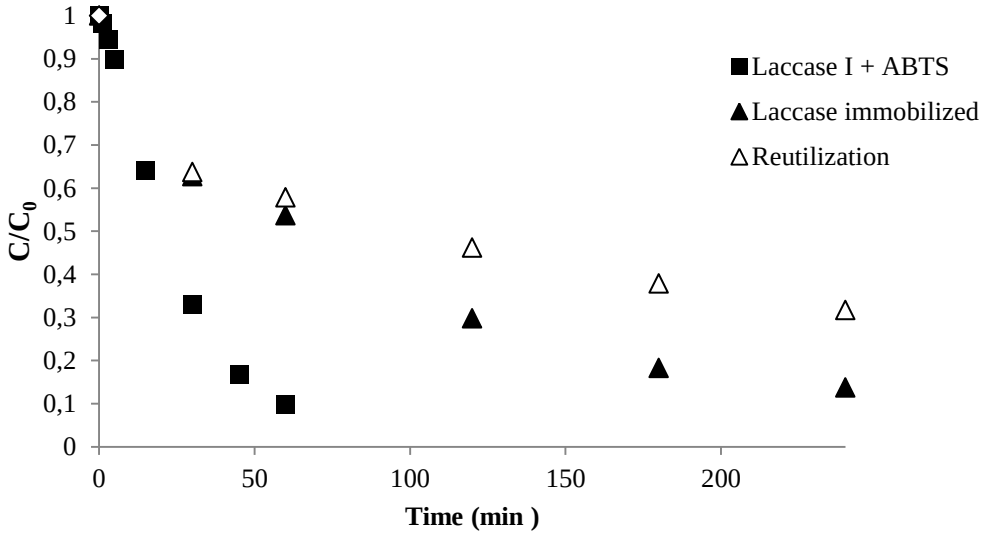

Fig. 28 - Phenol degradation using free and immobilized laccase.

5. CONCLUSION

According to the proposed objectives and the results it was possible to draw some conclusions.

Looking at the immobilization yield and recovered activity results it is possible to conclude that the best results were obtained for the oxidized MWCNTs oxidized with HNO_3 0.3M and post-treated with EDC/NHS, which leaded to an immobilization yield of 99.8% and 20.4% of recovered activity.

With respect to thermal stability, and comparing various treatments, the approach with the best results was also obtained for MWCNTox_0.3M+EDC/NHS. For the enzyme immobilized in this material, at 60 °C, a α value of 63.04 was obtained, which is almost two times higher than the values obtained for the other materials tested. The enzymatic activity for this laccase-bioconjugate towards ABTS oxidation was 65% after 4 hours. Comparing with free laccase, better results were obtained for immobilized laccase concluding that enzyme immobilization improves the enzyme stability.

The functionalization that led to the best results in terms of reusability was, once again, the treatment with EDC/NHS. After 5 cycles the activity of the immobilized enzyme kept 65% of its initial value.

From the support characterization it was concluded that the treatment with nitric acid increases the surface area of the MWCNTs due to the creation of defect sites and/or holes on the sidewalls of the tubes. This treatment also promoted the introduction of oxygen containing functionalities such as carboxylic acids, and in a less extent anhydride, lactone and quinone groups at the MWCNTs surface. The FTIR-ATR results have also proved that the functionalization with EDC/NHS promotes the linkage between the support and the enzyme.

Free laccase and immobilized laccase can be used through a mediated system with ABTS to degrade phenol. With the free enzyme, degradation process occurs more rapidly, resulting in a degradation of 97% after 1 hour. For the immobilized enzyme, the process is slower, with a phenol degradation of 87% being obtained after 4 hours of reaction. However, it was found that it is possible to reuse the enzyme-support complex obtaining a percentage of 70% of phenol degradation after reuse.

Overall, it can be concluded that the treatment with EDC/NHS is very promising for the efficient and stable immobilization of laccase and for being used in biocatalytic processes. In relation to the phenol degradation, it was verified that it is possible to degrade phenol using immobilized laccase and the possibility of reutilization brings enormous advantages in terms of process costs and environmental protection.

6. FURTHER RESEARCH AND FINAL APPRECIATION

6.1. Further research

During this work it was verified that it would be interesting to further investigate some aspects dealing with biocatalytic process optimization and enzyme characterization.

Tests as variation in pH, concentration of reagents and immobilization time were some examples of what could be done to optimize the biocatalytic reaction using the materials prepared through the various functionalization approaches used in this work.

Moreover, due to the lower laccase activity observed after immobilization, it would be interesting to investigate the existence of changes in the enzyme structure. For this purpose, advanced techniques such as circular dichroism spectroscopy can be used allowing the determination of the change in the α and β conformations of the enzyme after utilization in the immobilized form.

With regard to phenol degradation using immobilized laccase, its optimization can be evaluated by varying the concentration of enzyme and/or mediator, pH and reaction time, looking for enhanced phenol degradation efficiency while reducing the reaction time. It would be interesting to determine the by-products of the reaction and try to monitor them in order to better understanding the phenol degradation mechanism. It can also be performed tests with other phenolic compounds and other mediators to realize the potential of this application.

Finally, the enzyme immobilization over a MWCNTs membrane using the adequate functionalization procedure would be a very interesting approach, which would allow the operation of biocatalytic processes in a continuous mode and without needing a separation step.

6.2. Final appreciation

This project evolved the development of competences in different research fields such as materials science, nanotechnology, and biotechnology. The results obtained during this project resulted from the synergies and complementarities of those areas. The surface modification of MWCNTs was performed using different approaches. Laccase was successfully immobilized and used in biocatalytic reactions. Finally, one promising method for laccase immobilization has been developed, resulting in a very active and stable bioconjugate with high potential for being used in biocatalysis applications.

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